Caspaselike Proteases Activated in Apoptotic Photoreceptors of Royal College of Surgeons Rats

Naomichi Katai, Takanobu Kikuchi, Hiroto Shibuki, Sachiko Kuroiwa, Jun Arai, Toru Kurokawa, and Nagahisa Yoshimura

PURPOSE. To study the role of caspase-like proteases, especially roles of more extensively characterized caspase-1 and caspase-2, in apoptotic photoreceptor cell degeneration in Royal College of Surgeons (RCS) rats.

METHODS. Both RCS and Sprague—Dawley rats were used. Cryosections of the retinas at various postnatal times were immunostained with antibodies against caspase-1 (interleukin-1β-converting enzyme, ICE) and caspase-2 (Nedd2/Ich-1). Double staining with TdT-dUTP terminal nick—end labeling (TUNEL), propidium iodide, and the antibodies was also performed. To evaluate the time course of protein expression, western blot analysis was carried out. The temporal profile of caspase-like protease activity was studied using a fluorogenic tetrapeptide substrate, acetyl-tyrosyl-valyl-alanyl-aspartic acid α (4-methyl-coumaryl-7-amide) (Ac-YVAD-MCA). Intravitreal injection of a caspase-1 inhibitor, acetyl-tyrosyl-valyl-alanyl-aspartic aldehyde (Ac-YVAD-CHO), at postnatal days 21 (P21) and P26 was performed to see if this caused a decrease in apoptotic cell number at P28.

RESULTS. TUNEL-positive photoreceptors of RCS rats stained strongly with antibodies against caspase-1 and caspase-2. Double staining studies revealed that caspase-1 and caspase-2 were coexpressed in apoptotic cells. Western blot analysis showed that active forms of caspase-1-like and caspase-2-like proteases were upregulated at P28, concurrent with the peak in TUNEL-positive cells. The enzymatic activity of caspase-1-like protease was elevated in RCS rat retinas at P28, and the inhibitor of caspase-1 transiently reduced the number of the apoptotic photoreceptors.


In Royal College of Surgeons (RCS) rats, photoreceptor degeneration caused by impaired phagocytosis of rod outer segments of the retinal pigment epithelial cells begins at about postnatal day 20 (P20), and almost all photoreceptors die out by P60. Recently, this retinal degeneration has been shown to be caused by apoptotic cell death, based on the finding that cells in the outer nuclear layer (ONL) are stained by TdT-dUTP terminal nick—end labeling (TUNEL) and that agarose gel electrophoresis of DNA extracted from the retina shows a typical ladder pattern.

Apoptosis is a phenomenon underlying normal development and many pathologic conditions and is achieved through activation of a cascade called a "death program." In general, the apoptotic process is divided schematically into five steps: activation, propagation, commitment, execution, and, finally, cell death. Interleukin-1β-converting enzyme (ICE)/caspase family proteins, identified by their homology with the nematode death gene ced-3, are executors of the apoptotic program in some vertebrate cells. Although the final step of apoptosis is invariably cell death, various signals contribute to the common pathway, and intermediate steps differ depending on the etiology of the apoptosis and cell type. If the apoptotic process has not proceeded beyond the execution step, a cell can be rescued in some instances by blocking expression of apoptosis-related genes or by overexpression of bcl-2.

Apoptosis plays a major role in the pathogenesis of many diseases in which photoreceptors degenerate, such as retinitis pigmentosa, retinal detachment, light injury, ischemic injury, and age-related macular degeneration. Therefore, it is important to know which genes are specifically expressed during apoptosis of photoreceptor cells. Herein, we will show that members of the caspase family may play a critical role in the apoptosis of photoreceptor cells in the RCS rat. In the present study, we concentrated on caspase-1 and caspase-2 because these enzymes are known to play an important role in neuronal apoptosis of ischemic brain injury and retinal development and apoptosis of rat pheochromocytoma-derived cell line cells (PC12).

MATERIALS AND METHODS

Animals

The care and maintenance of rats conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. RCS (Jcl-rdy/rdy) rats were obtained from Japan CLEA, Tokyo, Japan, and Sprague—Dawley (SD) rats from a local breeder. Rats were maintained under 12-hour light/12-hour dark conditions. RCS and SD rats of 14, 21, 25, 28, 33, 35, 45, and 56 days of age were used in this study.

Antibodies

Antibodies used in this study were obtained from various sources: Goat anti–caspase-1 (ICE) polyclonal antibody (M-19) and goat anti–caspase-2 (Nedd2/Ich-1) polyclonal antibody (N-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); fluorescein isothiocyanate (FITC)—and rhodamine-conjugated goat anti-rabbit IgG from DAKO (Glostrup, Den-
DNA nick end-labeling was performed according to a slightly modified method of Gavrieli et al.\textsuperscript{10} After rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg), they were perfused transcardially with 100 ml of normal saline supplemented with 1 U/ml heparin followed by 200 ml of 4\% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The eyes were enucleated and placed in 4\% paraformaldehyde for 24 hours at 4°C. They were rinsed with phosphate-buffered saline (PBS) and then transferred to 10\%, 15\%, and 20\% sucrose in 0.1 M PBS for 3 hours at room temperature. Samples were then snap-frozen in Tissue–Tek (Miles Laboratories, Elkhart, IN) on dry ice in hexane and stored at −80°C. Cryostat sections (10–15 μm) were thaw-mounted onto glass slides coated with poly-L-lysine and air-dried for 2 hours at room temperature. After cryosections were rinsed three times in 0.02 M PBS (pH 7.4), sections were incubated with biotinylated terminal dUTP transferase and biotinylated dUTP in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride) for 60 minutes at 37°C with terminal dUTP in the moisture chamber. After rinsing, the sections were reacted with avidin–FITC and examined with a Zeiss scanning laser confocal microscope (LSM 410 inverted Laser Microscope, Zeiss, Oberkochen, Germany). Positive controls were generated using DNase I in TdT buffer (1 μg/ml) before incubation with terminal transferase and biotinylated nucleotides. Propidium iodide (PI) staining was performed by incubating the sections with 50 μl PI (20 μg/ml) for 10 minutes at room temperature. TUNEL staining of cells, compacted nuclear staining, nuclear fragmentation, and loss of nuclear staining by PI were considered to be signs of apoptosis.

Immunohistochemistry

After rinsing with PBS, cryosections were incubated with 2\% normal goat or rabbit serum for 60 minutes at room temperature. Incubation with the primary antibody (anti-caspase-1, 10 μg/ml, and anti-caspase-2, 10 μg/ml) was carried out in a moisture chamber at 4°C overnight. FITC- or rhodamine-conjugated secondary antibody was reacted at room temperature for 2 hours.

Quantitative Analysis of TUNEL and Immunohistochemistry

At each point of the time course study for TUNEL and immunohistochemical staining, the number of TUNEL-positive and immunostained cells in the ONL was determined in 5 meridian sections through the optic nerve. The numbers of cells were counted in 0.4-mm lengths of the section 1 mm from the optic nerve head on the superior and inferior hemisphere using the Zeiss confocal microscope with the “measure” function. Results are expressed as mean ± SEM.

Analysis of Western Blots

Samples (n = 5 at each time) were homogenized in buffer containing Tris–HCl (pH 8.0), 250 mM NaCl, 0.5\% NP-40, 0.1 mM phenylmethylsulfonlfuryl fluoride, 10 μg/ml pepstatin, 20 μg/ml leupeptin, and 10 μg/ml aprotinin. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli.\textsuperscript{11} A 50-μg sample of protein was loaded on each lane, electrophoresed with 10\% acrylamide gel, and then transferred to nitrocellulose membranes. After being rinsed with Tris-buffered saline (pH 7.4) containing 0.25% Tween-20 (TBST), the membrane was incubated with the primary antibodies (0.5 μg/ml) in TBST for 1 hour at room temperature. After three washings, the membrane was incubated for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibody (Amersham, Buckinghamshire, UK) diluted 1:500 in TBST. The membrane was washed again and developed with the chemiluminescence ECL western blotting system (Amersham).

Intravitreal Administration of Caspase-1 Inhibitor

A specific inhibitor of caspase-1, acetyl-tyrosyl-valyl-alanyl-aspartate-aldehyde (Ac-YVAD-CHO), was obtained from the Peptide Institute (Osaka, Japan). A 20-nM solution of the Ac-YVAD in dimethyl sulfoxide was prepared, and 1 μl of the solution was injected into the vitreous cavity of the RCS rats at P21 and P26 by means of a 30-gauge needle. As a control, 1 μl dimethyl sulfoxide (vehicle) was injected in the same way. At P28, the eyes were enucleated, and the TUNEL staining was performed as described above.

Assay of Caspase Activity

Caspase-1–like protease activity was assayed as described by Nicholson et al.\textsuperscript{12} with slight modification.\textsuperscript{13} Briefly, resected retinas were homogenized in 10 mM Heps–KOH, pH 7.2, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonlfuryl fluoride, 10 μg/ml pepstatin, 20 μg/ml leupeptin, and 10 μg/ml aprotinin. Homogenates were centrifuged at 15,000 g for 30 minutes, after which the supernatants were transferred to new Eppendorf tubes. Aliquots of extracts (300 μg protein in 100 μl extraction buffer) were preincubated at 37°C for 30 minutes and then mixed with 5 μl of 10 mM tetrapeptide substrate, acetyl-tyrosyl-valyl-alanyl-aspartic acid α-(4-methyl-coumaryl-7-amide) (Ac-YVAD-MCA; Peptide Institute). Free aminomethylcoumarin (AMC) accumulation, which resulted from cleavage of the aspartate-AMC bond, was monitored in each sample at 37°C over 180 minutes using a spectrophotometer (Ultraspex III; Pharmacia, Cambridge, England). The absorbance of the each sample at 370 nm was plotted against time. Linear regression analysis of the velocity of each curve yielded the activity for each sample. Data were expressed as a percentage of the caspase-1–like activities in the samples compared to those in the P28 SD rat retina.

Statistical Analysis

The data were analyzed statistically by one-way ANOVA followed by Fisher’s post-hoc test. Probability values less than 0.05 were determined to be statistically significant.

RESULTS

TUNEL Staining

In both RCS and SD rats, TUNEL-positive cells were observed in the ONL at P14. In SD rats, these cells were no longer observed at
P21. In contrast, TUNEL-positive cells increased in number in the ONL of RCS rats at P21 to P45, with a peak at P28 (Figs. 1A, 1B). At P28, the number of TUNEL-positive cells was approximately 250/0.4-mm section, and about one third of the total cell number in the ONL at P28 was stained by TUNEL (Fig. 1B).

**Immunohistochemistry**

Specific staining of cells in the ONL of RCS rats was obtained using antibodies against caspase-1 and caspase-2 (Figs. 2A, 2B). In contrast, no specific staining was detected by any of the antibodies in the ONL of P28 SD rat retinas (graphic data not shown).

Diffuse cytoplasmic staining with anti-caspase-1 and anti-caspase-2 antibodies was seen in apoptotic cells that had condensed nuclei. In cells with weak or no PI staining, caspase-1-like and caspase-2-like immunoreactivities were observed in the nucleus and cytoplasm. Immunopositive cell species could not be identified because apoptotic cells had lost their original appearance. However, we concluded that the immunostained cells were apoptotic photoreceptors because of their location in the ONL and because they were not immunolabeled with anti-phosphotyrosine antibody (graphic data not shown).

**TUNEL-Positive and Immunostained Cells**

In RCS rats, quantitative analyses showed that the numbers of cells stained with antibodies against caspase-1 and caspase-2 were very similar during the follow-up period (Fig. 2C). Also, the time course of TUNEL-positive cells showed a similar pat-
tern of immunostaining. The numbers of immunostained cells were almost equal to that of TUNEL-positive cells at P14 and P21 (less than 6 cells/0.4-mm tissue section), but there were approximately 10 times more TUNEL-positive cells than immunostained cells at P28 (243.3 ± 6.8 TUNEL-positive cells/0.4-mm tissue section, 18.2 ± 6.0 caspase-1–positive cells and 13.7 ± 0.4 caspase-2–positive cells).

Double staining studies to identify TUNEL-positive cells and immunostaining using anti–caspase-1 and anti–caspase-2 antibodies showed that these signals were colocalized in the same cell (Figs. 2A, 2B). Caspase-1 and caspase-2 were also double-stained in the same cell (graphic data not shown). However, all the anti–caspase-1–positive cells were not always stained with anti–caspase-2 antibodies. The results of double staining with the antibodies and TUNEL staining showed that expression of caspase-1 and caspase-2 was correlated with photoreceptor apoptosis in RCS rats. Anti-phosphotyrosine antibody–labeled cells did not show coexpression of caspase-1 or caspase-2 (graphic data not shown).

**Immunoblot Analysis of Caspase-1– and Caspase-2–Like Proteases**

As shown in Figure 3A, anti–caspase-1 antibody depicted both 37.5-kDa and 20-kDa bands by western blot analysis. The apparent molecular weight of the bands agrees with that of caspase-1-like protease. Figure 3B shows that caspase-2–like protease is expressed in RCS rat retinas, based on the finding that anti–caspase-2 antibody blotted at both 40.5- and 33-kDa bands. The active form of caspase-1 and caspase-2 consists of long and short fragments that are derived from proteolytic processing of the proenzyme during apoptosis. Antibodies used in this study reacted with the active form of the 20-kDa subunit of caspase-1 and the 33-kDa active subunit of caspase-2. The level of expression of the 37.5 kDa caspase-1 subunit was constant, but that of the 20-kDa subunit, the active form, was upregulated at P21, P28, and P35 RCS rat. On the other hand, expression of the 40.5-kDa proenzyme was constant, whereas that of the 33-kDa active form of caspase-2 was upregulated at P14, P21, P28, and P35 in RCS rat retinas.
Assay of Caspase-1–Like Protease Enzymatic Activity

Activity of caspase-1–like protease was assayed using the specific tetrapeptide substrate. As shown in Figure 4, caspase-1–like protease activity was elevated in the extract from P28 RCS rat retinas. The levels of the activity were twofold those of P14 rats at P21 and sevenfold at P28. The time course of caspase activity changes was similar to those of immunohistochemical analysis and protein expression level of caspase-1 active form as shown in western blot analysis.

Effect of the Caspase-1 Inhibitor

Figure 5 indicates that the number of TUNEL-positive cells in ONL was significantly reduced in P28 RCS rat retinas injected with the inhibitor Ac-YVAD-CHO at P26. However, the number of TUNEL-positive cells of P28 rat retinas injected with the tetrapeptide at P21 was not decreased. These results indicate that the caspase-1 inhibitor can suppress, at least transiently, apoptosis of photoreceptor cells.

DISCUSSION

In this study, we demonstrate that caspase-1– and caspase-2–like proteases play an important role in photoreceptor apoptosis during retinal degeneration in the RCS rat. Activation of caspase-1–like protease contributes to photoreceptor apoptosis in the RCS rat.

Caspase-1 was first identified as the mammalian homologue of the \( \textit{ced-3} \) gene, initially identified in \textit{Caenorhabditis elegans}. Caspase-1 is known to promote apoptosis during development of the nematode, and, currently, 10 Ced-3–related cysteine proteases, termed caspase, have been cloned. These family members participate in one of two distinct signaling pathways: activation of proinflammatory cytokines and activation of apoptotic cell death. All the known caspase family proteases are synthesized as inactive proenzymes, which require cleavage to liberate one large and one small subunit to form the active enzyme. Activation of one caspase-like protease activity was elevated in the extract from P28 RCS rat retinas. The levels of the activity were twofold those of P14 rats at P21 and sevenfold at P28. The time course of caspase activity changes was similar to those of immunohistochemical analysis and protein expression level of caspase-1 active form as shown in western blot analysis.
can lead to cleavage and activation of another molecule of the same caspase, another caspase, or both, leading to an amplified apoptotic cascade. \(^4\) Indeed, in the Fas-mediated apoptosis of lymphoid cells, activation of caspase-3 via specific cleavage of the proenzyme by caspase-1 has been demonstrated. \(^7\) A number of experimental studies suggest that activation of the caspase family plays a critical role in the execution of apoptotic events. \(^3\)\(^7\)\(^9\) Caspase-1 was implicated in death caused by superoxide dismutase downregulation in PC12 cells but not in withdrawal of trophic factor support. \(^9\) Conversely, the antisense constructed to downregulate caspase-2 in PC12 cells inhibited cell death by withdrawal of trophic support but not oxidative stress. \(^7\) The activation of distinct caspases in the same cells thus can promote apoptosis initiated by the various stimuli. \(^7\)

In the present study, immunohistochemical analysis showed that caspase-1 and caspase-2 were colocalized in the same cell. Furthermore, on immunoblot analysis and measurement of enzymatic activities (Figs. 3, 4), the active forms of caspase-1- and caspase-2-like proteases were shown to be upregulated in P28 RCS rat retinas. These results suggest that caspase-1 and caspase-2 are correlated with the photoreceptor apoptotic process in RCS rats. However, it remains to be determined how these two caspases are activated in photoreceptor degeneration. To estimate the potential role of caspase-1-like protease in photoreceptor apoptosis more directly, we examined in vivo effects of a specific inhibitor of caspase-1 on the number of apoptotic photoreceptors. When administered into the vitreous, Ac-YVAD-CHO reduced the number of apoptotic nuclei for long periods reflects a failure of phagocytosis.

To our knowledge, this is the first report of a correlation between photoreceptor apoptosis and the caspase family. Although this study suggests an important role for caspase-1 and caspase-2 in the process of photoreceptor apoptosis in the RCS rat retina, further studies are necessary to clarify the molecular mechanism underlying retinal degeneration in these animals.

References


VEGF Increases Retinal Vascular ICAM-1 Expression In Vivo

Ming Lu, Victor L. Perez, Naili Ma, Kazuaki Miyamoto, Hai–Bing Peng, James K. Liao, and Anthony P. Adamis

PURPOSE. Intraocular injections of vascular endothelial growth factor (VEGF), a peptide implicated in the pathogenesis of diabetic retinopathy, can induce retinal ischemia. Diabetic retinal ischemia may be caused, in part, by the adhesion of leukocytes to the retinal vasculature. In this study, the ability of VEGF to increase the expression of intercellular adhesion molecule-1 (ICAM-1) and other adhesion molecules in capillary endothelium and the retinal vasculature was examined.

METHODS. The expression of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin on human brain capillary endothelial cell monolayers exposed to VEGF was quantitated by immunoassay. The effect of VEGF on retinal vascular ICAM-1 expression was determined in ICAM-1 immunofluorescence studies of retinal flat-mounts and in RNase protection assays.

RESULTS. VEGF increased capillary endothelial cell ICAM-1 levels in a dose- and time-dependent manner (6–24 hours, plateau after 6 hours; EC50, 25 ng/ml). VEGF failed to alter E-selectin, P-selectin, or VCAM-1 levels under the conditions tested. Intravitreal injections of pathophysiologically relevant concentrations of VEGF increased ICAM-1 protein and mRNA levels in the retinal vasculature.


Vascular endothelial growth factor (VEGF) is a major mediator of retinal ischemia-associated ocular neovascularization. Intraocular VEGF levels have been temporally and spatially correlated with neovascularization,1 and the specific inhibition of VEGF in animal models has been shown to suppress the neovascularization of the iris2 and the retina.3 More recently, exogenous VEGF has also shown to induce many of the angiographic and histopathologic features of diabetic retinopathy in nonhuman primates, including retinal ischemia.4 The cellular and molecular mechanisms underlying this phenomenon have not been defined.

Fluorescein angiographic studies in humans have demonstrated that some diabetic retinal ischemia is reversible.5 However, in most eyes a large proportion of the diabetic retinal ischemia that develops is irreversible and results from the formation of acellular capillaries.6 Diabetic retinal leukostasis7 may be operative in the development of both types of retinal ischemia. Increased numbers of monocytes and granulocytes have been demonstrated to occupy the lumens of retinal capillaries and postcapillary venules in diabetic rats, with some leukocytes lying adjacent to dying endothelial cells.8 Increased numbers of granulocytes have also been demonstrated in the retinas of diabetic humans.9 One potential mechanism mediating this phenomenon is the increased adherence of leukocytes to the vascular endothelium. Intercellular adhesion molecule-1 (ICAM-1) mediates the adhesion of neutrophils and monocytes to vascular endothelium, and increased ICAM-1 levels have been found in the retinal vasculature of human diabetics.9 In addition, human diabetic neutrophils have been shown to be more prone to upregulate ICAM-1 ligand CD18.10 Because of these observations, studies were undertaken to characterize the effect of VEGF on the expression of endothelial cell adhesion molecules in vitro and in retina in vivo.

METHODS

Cell Culture

Human brain capillary endothelial cells (HBCEC) were obtained from Cell Systems Corporation (Kirkland, WA) and maintained in medium 199 (GIBCO BRL, Gaithersburg, MD), containing HEPES (25 mM), heparin (1%), endothelial cell growth factor (50 µg/ml), L-glutamine (2 mM), 100 U/ml penicillin, 100 µg/ml streptomycin, and 15% fetal bovine serum (HyClone Laboratories, Logan, UT) according to the manufacturer’s instructions. For antibody immunoassay (EIA), HBCECs were grown on tissue culture–treated plastic microtiter 96-well plates, coated with 0.1% (wt/vol) gelatin (Difco, Detroit, MI), and allowed to reach confluence.

Adhesion Molecule Quantitation via EIA

Endothelial cell adhesion molecule levels were measured by EIA in HBCECs. HBCECs were grown to confluence in 96-well plates and treated with serum-free medium with or without various concentrations of recombinant human VEGF165 (a gift of Napoleone Ferrara, Genentech, South San Francisco, CA; endotoxin < 0.03 EU/ml) or recombinant human tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis MN; positive control) for 24 hours. Cell surface EIAs were completed using mouse anti-human monoclonal antibodies against ICAM-1 (Ab HIU 5/3), VCAM-1–1 (Ab E1/6), and E-selectin (Ab H18/7).11 The EIAs were carried out by incubating monolayers first with saturating concentrations of specific monoclonal antibodies against the target molecule, followed by biotinylated goat anti-mouse IgG, and finally with streptavidin–alkaline phosphatase. The surface expression of each protein was quantified spectrophotometrically, reading the optical density of the wells (410 nm) 15 to 60 minutes after the addition of a chromogen (p-nitrophenylphosphate), as described previously.11 The data

From the 1Laboratory for Surgical Research, Children’s Hospital, Boston, Massachusetts; the 2Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts; the 3Schepens Eye Research Institute, Boston, Massachusetts; and 4Brigham and Women’s Hospital, Boston, Massachusetts. These authors contributed equally to the work presented here and should therefore be regarded as equivalent senior authors. Supported by the National Eye Institute (APA), the Massachusetts Lions, and the Roberta W. Siegel Fund (APA).


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Reprint requests: Anthony P. Adamis, Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114.
were normalized against protein concentrations and expressed as mean ± SEM.

**ICAM-1 Immunofluorescence**

Animals were cared for in accordance with the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research. The experiments were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Normal C57B/6 mice (male, 6 to 8 weeks old) were examined for retinal ICAM-1 expression after intravitreal injections of recombinant murine VEGF164 (R&D Systems, Minneapolis, MN) or vehicle alone (n = 6; endotoxin < 0.1 EU/mg). After anesthesia with a mixture of ketamine (Ketalar; Parke-Davis, Morris Plains, NJ; [150 mg/kg]) and xylazine (Rompun; Harver-Lockhart, Morris Plains, NJ; [60 mg/kg]), 2 µl concentrated VEGF or phosphate-buffered saline (PBS) solvent was injected slowly into the vitreous cavity to attain a final concentration of 100 ng/ml VEGF. After 24 hours, the animals were killed by cervical dislocation, and the eyes were enucleated and immersion-fixed in 0.5% (wt/vol) paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 minutes. The intact retinas were dissected out under a surgical microscope as previously described and further fixed in 70% ethanol at room temperature for an additional 30 minutes. The retinas were washed in 0.1 M PBS (pH 7.4) with 0.1% (vol/vol) Triton X-100 for 30 minutes and then incubated with biotinylated anti-mouse ICAM-1 (1:50 diluted in 0.1 M PBS containing 1% [wt/vol] bovine serum albumin, BSA) overnight at 4°C. After washing at room temperature with PBS containing 1% BSA, the retinas were further incubated with streptavidin–fluorescein isothiocyanate (1:100 diluted in PBS containing 1% BSA; Amersham, Arlington Heights, IL) for 2 hours at room temperature. An isotype-matched IgG was used as a negative control. The retinas were co-stained with 2 ng/ml ethidium bromide for 10 seconds and mounted. Endothelial cell nuclei were counted per unit length of vessel.

**RNase Protection Assay for ICAM-1**

The experiments were approved by Children’s Hospital Animal Care and Use Committee. Male Sprague–Dawley rats weighing 200 to 250 g were anesthetized with 0.1 mg/kg sodium amobarbital. Pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride (Alcon, Humancao, Puerto Rico). Murine VEGF164 or human TNF-α, in a total volume of 5 µl PBS, was injected into the right vitreous at a site 1 mm posterior to the limbus using a Hamilton syringe with a 30-gauge needle. The final concentrations of VEGF and TNF-α were calculated to be 100 and 10 ng/ml, respectively. The contralateral control eyes received 5-µl PBS injections. The eyes were enucleated 2.5 hours later. The rats were euthanized with an anesthetic overdose followed by CO2 incubation. All injections were done under direct observation using a surgical microscope. Any eyes that had damage to the lens or retina were not used for analyses. The retinas were gently dissected free and cut at the disc. The tissue was placed in an Eppendorf tube, snap-frozen in liquid nitrogen, and stored at −80°C. The retinas were homogenized in 1 ml RNAzol (Biotex Laboratories, Houston, TX) at 4°C and prepared for RNase protection assays.

The ICAM-1 riboprobe was produced by subcloning the coding sequence of the rat ICAM-1 cDNA into the EcoRI–BamHI site of the pBluescript II KS vector. Transcription by T7 RNA polymerase after linearization by EcoRI resulted in a probe of 225 nucleotides (nt). This probe protects a 166-nt fragment of ICAM-1. All samples were simultaneously hybridized with an 18S riboprobe (Ambion, Austin, TX) to normalize for variations in loading and recovery of RNA. Full-length protection of this probe results in an 80-nt fragment. The assay was performed as previously described. Ten micromolars of total cellular RNA was hybridized with 32P-labeled antisense ICAM-1 and 18S riboprobes (200,000 cpm of each) overnight at 42°C in 30 µl hybridization buffer. Hybridized RNA was digested with nuclease P1 (20 µg/ml) and RNase T1 (2 µg/ml) for 1 hour at 25°C in 300 µl digestion buffer. Digestions were terminated by the addition of 20 µl of 10% sodium dodecyl sulfate and 50 µg proteinase K for 15 minutes at 57°C. After phenol/chloroform extraction, the RNA was ethanol precipitated and re-hybridized with a 18S riboprobe at 42°C for 1.5 hours. The protected bands were visualized by autoradiography and quantified with a Packard Betascope. The ICAM-1 expression was normalized against 18S riboprobe

**Figure 1.** Time (A) and dose (B) responses of surface ICAM-1 levels in HBCECs after exposure to serum-free medium with and without VEGF. Time course experiment was performed using 50 ng/ml VEGF. Dose response of ICAM-1 (B) was performed after a 24-hour exposure to serum-free medium with and without VEGF. *P < 0.05 vs control, n = 8. Data are expressed as percentage over control (no VEGF treatment) optical density, normalized against protein concentration in each well.
extraction and ethanol precipitation, the protected fragments were resolved on 6% polyacrylamide, 7 M urea gels and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistics
Significance was calculated using the paired Student’s t-test. P < 0.05 was deemed significant.

RESULTS
VEGF increased the expression of ICAM-1 on HBCEC in a time- and dose-dependent manner (6–24 hours, plateau after 6 hours; EC_{50}, 25 ng/ml) (Figs. 1A, 1B). Expression levels of surface VCAM-1 (Fig. 2A), E-selectin (Fig. 2B), and P-selectin (Fig. 2C) were unaffected by incubation with VEGF_{165} for 6 hours (data not shown) or 24 hours. Incubation of HBCEC monolayers with TNF-α (positive control) produced significant increases in VCAM-1 (2.4 ± 0.1-fold, n = 8, P < 0.05 versus control), E-selectin (17.5 ± 1.3-fold, n = 8, P < 0.05 versus control), P-selectin (10.7 ± 1.2-fold, n = 8, P < 0.01, data not shown), and ICAM-1 (2.5 ± 0.2-fold, n = 8, P < 0.05 versus control, data not shown).

Compared to the PBS-injected eyes, the VEGF-injected eyes had a marked homogeneous upregulation of ICAM-1 immunoreactivity, particularly in the mid-peripheral retina and the postcapillary venules. There was also a moderate upregulation in the arterioles and capillary beds (Fig. 3). Because VEGF is an endothelial cell mitogen, we examined whether VEGF increased endothelial cell numbers, possibly confounding the increases in ICAM-1 expression. Twenty-four hours of VEGF treatment did not significantly increase the number of endothelial cell nuclei per fixed segment length in the retinal vasculature (data not shown).

To examine whether VEGF increases ICAM-1 expression at the transcript level, RNase protection assays of rat retinal RNA were performed after intravitreal injections of VEGF or PBS buffer. Compared to the PBS-injected eyes, the VEGF-injected eyes had a 2.1 ± 0.4-fold (n = 4, P < 0.05) upregulation of ICAM-1 mRNA (Fig. 4). As a positive control, TNF-α induced a 3.1 ± 0.8-fold increase of ICAM-1 mRNA (n = 5, P < 0.05).

DISCUSSION
These data demonstrate that VEGF can increase ICAM-1 expression on capillary endothelial cells in vitro and in the retinal vasculature in vivo. The concentration of VEGF required to increase ICAM-1 in vitro (EC_{50}, 25 ng/ml) is comparable to that measured in the vitreous of eyes with retinal ischemia and neovascularization.1 The VEGF-induced effects appear to be specific for ICAM-1 under the conditions tested. These data are consistent with those of Melder and associates14 showing an increase in bioactive ICAM-1 in angiogenic tumor endothelium in response to VEGF. However, our data differ regarding the VEGF-induced upregulation of VCAM-1, because it was not observed in our system. One reason for these divergent results may be the different types and strains of endothelial cells used in our studies.

In addition to VEGF, other pathophysiologically relevant stimuli may increase ICAM-1 in the diabetic retinal vasculature.
Factors relevant to diabetes, such as the cytokine TNF-α, have been shown to increase ICAM-1 in other systems. TNF-α levels are increased in the serum of diabetics, and as confirmed by our data, TNF-α is a potent inducer of endothelial cell ICAM-1 expression. As in other tissues, the induction of ICAM-1 may be causal for the leukostasis observed in the diabetic retina. In the brain, ICAM-1 mediates leukocyte adhesion to postcapillary venules and is associated with capillary occlusion and vascular endothelial cell damage.

Retinal VEGF levels appear to be elevated early in diabetest, before there is any histopathologic evidence of retinal ischemia. This makes it more likely that endogenous VEGF serves to increase retinal vascular ICAM-1 in vivo. We hypothesize that VEGF, in whole or in part, increases retinal ICAM-1 expression and that ICAM-1 mediates the binding of leukocytes to the vasculature, accounting for the early reversible phase of retinal ischemia. With time, the retinal leukostasis becomes chronic and widespread, hastening the death of endothelial cells and pericytes. With the development of more widespread ischemia, retinal VEGF levels are further upregulated, triggering greater ischemia and ultimately neovascularization. This hypothetical sequence of events remains to be proven. Proof requires the specific inhibition of VEGF and ICAM-1 in the diabetic retina and the direct monitoring of its functional and anatomic consequences. These studies are underway.

**References**


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**β-Arrεstin–Related Proteins in Ocular Tissues**

Corine Nicolas–Léveque, Ibtissem Ghedira, Jean Pierre Faure, and Massoud Mirshahi

**PURPOSE.** Proteins of the arrestin family contribute to the regulation of G-protein-mediated transduction. In this study, the presence of β-arrestins in ocular tissues was investigated.

**METHODS.** Mouse monoclonal and rabbit polyclonal antibodies were raised against the peptide Val-Asp-Thr-Asn-Ile-Leu-Glu-Leu-Asp-Thr-Asn-Asp-Asp-Ile, a sequence present in β-arrestins 1 and 2 but absent from visual arrestin. These antibodies were used for the immunohistologic detection of β-arrestins in paraffin sections of rodent eyes fixed in Bouin’s solution. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of RNA from bovine retina, retinal pigmented epithelial (RPE) cells, lens epithelial cells, and human corneal fibroblasts was performed using β-1 arrestin primers.

**RESULTS.** In the eye, β-arrestin staining predominated in RPE, inner segments of photoreceptors, synaptic spherules of rods, inner plexiform layer and ganglion cell fibers, epithelial cells from ciliary body, and vessels. RT-PCR amplified a 480 bp product, corresponding to the predicted length. The sequence of PCR products from bovine retina and RPE cells was identical with the bovine β-arrestin mRNA.

**CONCLUSIONS.** β-arrestins were detected in several ocular tissues. In photoreceptor cells, their specific localization in the synaptic terminals and plexiform layer suggests a role of β-arrestin in synaptic transmission. In other ocular tissues, the presence of β-arrestin may be related either to adrenergic signal transduction or to signal transduction mediated by other G-protein–coupled receptors. (*Invest Ophthalmol Vis Sci.* 1999;40:1812–1818)

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Proteins of the arrestin family are implicated in the desensitization of the seven transmembrane domain receptors, a large family of membrane proteins that are at the origin of several transduction cascades mediated by G proteins. The most extensively studied processes implicating these molecules include the visual transduction cascade in the retina and optic nerve of human subjects with nonproliferative diabetic retinopathy.

Consequently, we investigated the presence of β-arrestin in ocular tissues by RT-PCR analysis and by immunohistochemi-
istry. The results are compatible with the idea that β-arrestins are widely distributed in a number of ocular tissues.

**METHODS**

**Preparation of Monoclonal Antibodies**

The peptide Val-Asp-Thr-Asn-Ile-Leu-Glu-Leu-Asp-Thr-Asn-Asp-Asp-Ile which represents an amino acid sequence present in β-arrestins 1 and 2 but absent from visual arrestins was coupled to keyhole limpets hemocyanin (KLH) according to the method described by the manufacturer (Pierce, Rockford, IL). Mouse monoclonal antibodies (mAbs) were raised against this peptide as previously described by de St. Groth and Scheiderg."9 Several cell lines were selected based on the anti-β-arrestin peptide, and anti-retinal arrestin activity in their supernatants was analyzed by enzyme-linked immunosorbent assay (ELISA). The positive cells were expanded and cloned by the limiting-dilution technique. Aliquots of antibody-secreting clones were kept frozen at

**Polyclonal Rabbit Antiserum to the β-Arrestin Peptide**

Animals used in these experiments were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Two hundred micrograms of the peptide coupled to KLH, was reconstituted in Freund's complete adjuvant and injected intradermally at four foci into adult fawn Burgundy rabbits. A booster was administered 3 weeks later, and the serum was tested for anti-β-arrestin activity by ELISA. To assess antibody specificity, increasing dilutions of the supernatants (β10D3 and β1D4), and of the anti-β-arrestin serum, were incubated (48 hours, 4°C) with either 40 μg free peptide or visual arrestin (antigen-S). After ultracentrifugation, the supernatants were tested for their anti-β-arrestin activity by ELISA. β10D3 mAb was finally selected for immunoanalysis of β-arrestin in ocular tissues. The antibody absorbed by the peptide (800 μg peptide/ml β10D3 supernatants) was used as a control.

**Cell Culture**

Bovine enucleated eyes, obtained from the local abattoir, were stored for 24 hours at 4°C to reduce adhesion between the neural retina and the pigmented epithelium and were bisected posterior to the ora serrata. After removal of the vitreous body and separation of the retina, the eyecup was rinsed with RPMI-1640 (Gibco, Paisley, Scotland, UK), and 1 ml trypsin (0.25%, Gibco) was introduced into the eyecup, followed by
incubation for 1 hour at 37°C. The retinal pigmented epithelium (RPE) cells were pipetted out of the Bruch's membrane, washed with RPMI by centrifugation (10 minutes, 1500 g), and finally resuspended in RPMI 1640 supplemented with 5 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 20% fetal bovine serum. The cells were seeded at 5 x 10^4 cells/cm^2 in tissue culture flasks and incubated at 37°C in 5% CO_2 atmosphere. The RPE cells were used after the fourth passage.

Epithelial cells of the bovine lens were cultured according to Plouet et al., and were kindly provided by Yve Courtois, INSERM, Paris. Normal human corneal fibroblasts were isolated from peripheral cornea, as previously described, and cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, 5 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in 5% CO_2 atmosphere. The RPE cells were used after the fourth passage.

Western Blot Analysis

Bovine retina or cultured RPE cells were homogenized in 10 mM phosphate buffer (pH 7.7) containing a mixture of anti-proteases, as previously described. Cell-free extract was obtained by centrifugation at 105,000g (1 hour, 4°C). Soluble extracts of bovine retina or RPE were electrophoresed in 10% sodium dodecyl sulfate (SDS) acrylamide gel, then electrotransferred to polyvinylidene difluoride membranes. The membrane were saturated in 15% nonfat dry milk diluted in phosphate-buffered saline (PBS), washed, and incubated either with the mAb (culture supernatant diluted 1:5) or the rabbit antisera (diluted 1:500) directed against b-arrestin, for 18 hours at 4°C. Antigen–antibody complexes were detected by successive incubations with sheep anti-mouse or anti-rabbit IgG-biotinylated antibody (diluted 1:500) for 2 hours followed by streptavidin-biotinylated horseradish peroxidase complex (diluted 1:500) for 1 hour. Finally, the immunoperoxidase was developed with 4-chloro-1-naphthol (0.05%) and H_2O_2 (0.01%). The blots were washed three times with PBS between each of the successive steps.

Animal Experimentation

Prague virol glaxo rats (X5) were exposed to 500 lux and killed after 40 minutes One eye from each animal was immediately immersed in Bouin's fixative and embedded in paraform wax for immunohistochemistry. The retina was dissected from the other eye and kept frozen at -80°C for PCR analysis.

Immunohistochemistry

Thin paraffin sections were deparaffinized with toluene and incubated successively with the mAbs (undiluted supernatants), 1:50 diluted biotinylated anti-mouse IgG sheep antibody (Amersham, Amersham, UK), and streptavidin-fluorescein isothiocyanate complex (1:100, Amersham). All reagents were diluted in PBS containing 1% bovine serum albumin. Each incubation step (60 minutes at room temperature) was followed by extensive washing with PBS. The slides were then mounted (Fluoprep, Biomérieux, Marcy-l’Etoile, France), and sections were photographed (400 ASA film; Fuji, Tokyo, Japan) under a fluorescence microscope (Nikon, Tokyo, Japan).

RNA Preparation

Cell RNA (from bovine retina, RPE cells, lens epithelial cells, and human corneal fibroblasts) was prepared using reagent according to the protocol provided by the manufacturer (Trizol Gibco). It consisted of an improvement over the single-step RNA isolation method developed by Chomczynski and Sacchi. One microgram total RNA was used for reverse transcription into cDNA. RNA was denatured for 3 minutes at 65°C and incubated for 60 minutes at 4°C in a final volume of 20 μl in the presence of 0.5 mM dNTP (Pharmacia, Uppsala, Sweden), 0.01 M dithiothreitol, 0.5 μg oligo-dT(18) (Promega, Madison, WI), 200 U reverse transcriptase (Superscript RNase H–Gibco) and 1 U RNASin (Promega) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl_2. This mixture was then heated at 95°C for 5 minutes.

Polymerase Chain Reaction

Ssense and antisense oligonucleotide primer pairs were synthesized to match the sequences of b-arrestin 1 (forward 5’-CTATGTCGGACAGCCCTTG-3’; reverse 5’-ACTTCTGAGGCTTTGCTG-3’) and b actin (forward, 5’-CTCGAGAAGAGCTATGAGCTG-3’; reverse 5’-AATCTCCTTCTG-3’).
PCR was performed using 6 μl cDNA. The assay mixture contained 2.5 U Taq DNA polymerase (Gibco), 200 μM dNTP, 2 μM respective oligonucleotide primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂ in a volume of 50 μl. The mixture was overlaid with mineral oil (Sigma) and amplified in a thermal cycler (Crocodile III; Appligene, Illkirch, France). Denaturation was carried out at 93°C for 1 minute (3 minutes in the first cycle) followed by an annealing step at 55°C or 61°C (depending on the primers) for 1 minute and an extension step at 72°C for 1 minute (10 minutes in the last cycle) to a total of 25 cycles for β-actin and 35 cycles for β-arrestin. The PCR products (10 μl) were electrophoresed on 1.2% agarose (Gibco) gels containing ethidium bromide in 45 mM Tris-borate and 1 mM EDTA (TBE buffer). A negative H₂O control was amplified under the same conditions. PCR products were sequenced by a method that combines limited chemical degradation of 3' fluorescent-labeled DNA with densitometric analysis (model 377 automatic sequencer; Applied Biosystems, Foster City, CA).

**RESULTS**

The peptide VDTNLIELTNDLI represents a sequence specific to β-arrestin and absent from the visual arrestin (Fig. 1). Monoclonal antibodies directed against this peptide linked to KLH, were selected by the cell lines β10D3 and β1D4, whereas polyclonal antibodies were raised in the rabbit (Fig. 2). The specificity of the antibody was confirmed by incubating various dilutions of antisera or mAbs with 40 μg peptide. Incubation with this peptide, but not with S-antigen, neutralized the antibody progressively, and total elimination was observed with a 1:128 dilution of mAb β10D3 (similar results were obtained with mAb β1D4). A strong decrease of immunoreactivity was also observed with the 1:1280 dilution of the antiserum. Thus, the antibody appeared to be specifically directed against the β-arrestin peptide antigen (Fig. 2). In concurrent experiments, these antibodies did not react with retinal arrestin in the ELISA (results not shown).

In western blot analysis, bovine retinal or RPE extracts were resolved as a unique band of 48 to 50 kDa, both with the...
rabbit antiserum (Fig. 3A) and with mAbs β10D3 and β1D4 (Fig. 3B). This molecular weight is in good agreement with β-arrestin reported previously.¹⁰

Immunohistochemistry, with the mAb reveled that β-arrestin was present in both segments of the photoreceptor (rod and cone cell layer) the inner segment, the synaptic spherules of rods, and in the outer segments (arrow). Similarly, β-arrestin was present in fibers and granules of outer

**Figure 5.** Immunofluorescent staining of the pars plana and pars plicata of the ciliary body, the iris, and the RPE. The nonpigmented (arrow) and pigmented (double arrow) epithelia exhibited β-arrestin–specific immunofluorescence in the pars plana (A) and pars plicata (B) of the ciliary body. In the iris (C) and choroid (D) only the vessel walls were stained; no immunoreactivity was observed in the epithelium of the iris (C, asterisk). Strong immunoreactivity was observed in the RPE (D, arrow). The β-arrestin-specific immunoreactivity was intense in the cytoplasm of the cells. Original magnification, ×200, section from PVG rat eye. Immunofluorescence reaction with mAb β10D3.
and inner plexiform layers, and in the ganglion cell layer (Fig. 4C). Intense staining was also observed in the ciliary body, iris, pars plana (Fig. 5A), and pars plicata (Fig. 5B), where both the unpigmented (arrow) and the pigmented (double arrow) epithelia were immunopositive. In the iris (Fig. 5C), only the vessel wall was stained by these antibodies, whereas no immunoreactivity was observed in the epithelium of iris (Fig. 5C, asterisk). Finally, β-arrestin was also detected in the cytoplasm of RPE cells (Fig. 5D, double arrows) and in the endothelial cells of vessels in the choroid (Fig. 5D, arrow). Similar results were observed with the polyclonal rabbit antiserum (results not shown).

To determine the levels of mRNAs for β-arrestin 1 and β-actin, PCR was performed with RNA prepared from different ocular tissues. Data in Figure 6 show that the PCR products corresponded to the predicted lengths, according to the primers used for amplification. The mRNA for β-arrestin was present in bovine retina, bovine RPE cells, bovine lens epithelial cells, and human corneal fibroblasts. The DNA sequences for β-arrestin from retina and RPE were determined by the use of four different chain terminators in a single electrophoretic run. Significant homology was found among amino acid sequences of β-arrestin from bovine retina and RPE, bovine retinal arrestin, and bovine β-arrestins 1 and 2 (results not shown). These provide clear evidence for β-arrestin-like proteins in ocular tissues.

DISCUSSION

The sequence of the peptide used for the preparation of the mAbs to β-arrestins is common to β-arrestins 1 and 2 but absent from other known mammalian arrestins. Therefore these mAbs can specifically detect β-arrestins and possibly other still unknown closely related arrestins. They do not detect the S-antigen (the arrestin involved in phototransduction) or the cone (or X-)-arrestin17,18 that are expressed in the retinal photoreceptor cells. The mRNAs for β-arrestins 1 and 2 have previously been detected in the retina and in many other nonocular tissues by PCR.19 To our knowledge, immunodetection of these proteins in the eye has not been previously reported. Attramadal et al.20 showed the presence of β-arrestins 1 and 2 in sections of rat brain in synaptic junctions and in some neurons.

We show here that, in the retina, β-arrestins were specifically located in the synaptic spherules of photoreceptor cells. They were also present in the inner segments, the probable site of their synthesis. Furthermore, β-arrestins were also detected in other synaptic bodies and nerve fibers of the inner retinal layers. This localization suggests that these molecules have a role in synaptic transmission in the retina. Until now, adrenergic receptors β,21 β-2,22-25 and β 1, 224 were reported in the retina, but the absence of these receptors25 also has been reported. Although the cellular localization of these receptors is unknown, the α-adrenergic receptor also was described in the retina.26 The β-adrenergic receptor kinases 1 and 2, which phosphorylate the β-adrenergic receptor and thus promote the binding of β-arrestin to the receptor, are widely distributed in the brain synapses.20 These kinases also are able to phosphorylate other receptors. This suggests that β-arrestins could similarly desensitize other receptors involved in neurotransmission. However, the specificity of each arrestin for the recognition of individual G-protein–coupled receptors remains to be explored.

β-Arrestin-related proteins may be implicated in synaptic receptor desensitization, but they also could be an adaptor for the adrenergic-related receptors involved in endocytosis of corresponding receptors.7 Exocytosis, another process in the synaptic function, also is dependent on guanosine triphosphate–binding proteins and arrestin-related proteins may be implicated in this manner.

The presence of β-arrestins in the epithelial cells in the eye is in agreement with the localization of adrenergic receptors described in ocular tissues.8 Consequently, these proteins are likely to play an important role in the function of adrenergic receptors.

References

6. Ferguson SS, Downey WE, Colapietro AM, Barak LS, Menard L, Caron MG. Role of beta-arrestin in mediating agonist-promoted G

Screening of the Gene Encoding the α'-Subunit of Cone cGMP-PDE in Patients with Retinal Degenerations

Yong Qing Gao, Michael Danciger, Reid Longmuir, Natik I. Piriev, Dan Yun Zhao, John R. Heckenlively, Gerald A. Fishman, Richard G. Welleber, Samuel G. Jacobson, Edwin M. Stone, and Debora B. Farber

Purpose. To screen the exons of the gene encoding the α'-subunit of cone cyclic guanosine monophosphate (cGMP)-phosphodiesterase (PDE6C) for mutations in a group of 456 unrelated patients with various forms of inherited retinal disease, including cone dystrophy, cone–rod dystrophy, macular dystrophy, and simplex/multiplex and autosomal recessive retinitis pigmentosa.

Methods. The 22 exons of the PDE6C gene were screened for mutations either by denaturing gradient gel electrophoresis and single-strand conformation polymorphism electrophoresis (SSCP) or by SSCP alone; variants were sequenced directly.

Results. Although many sequence variants were found, none could be associated with disease. Conclusions. The results show that PDE6C was not the site of the mutations responsible for the types of inherited retinal degenerations analyzed in the large population of patients in the present study. The types of degeneration included those that predominantly affect cone-mediated function (cone and cone–rod dystrophies) or rod-mediated function (retinitis pigmentosa) or that have a predilection for disease in the macula (macular dystrophies).

In mammalian rods the visual response is induced by a cascade of events mediated by rhodopsin, rod transducin, and rod cyclic guanosine monophosphate-phosphodiesterase (cGMP-PDE). Activated rod cGMP-PDE breaks down cGMP causing the cGMP-gated cation channels to close, bringing about an eventual alteration in the flow of neurotransmitter from the rod synaptic terminal. The same basic cascade most likely occurs in the cone visual cell. However, the proteins making up the cascade are different. For example, instead of rhodopsin, one of the cone opsins is present, and instead of one α- and one β-catalytic subunit in rod cGMP-PDE, there are two identical catalytic α'-subunits in cone cGMP-PDE.

Retinal degenerations such as retinitis pigmentosa (RP) have been associated with mutations in several genes including those for both the α- and β-subunits of rod cGMP-PDE (PDE6a and PDE6b). 2–5 Mutations in these genes are associated with autosomal recessive (AR) RP, and they were detected by exon screening of large numbers of unrelated patients. Therefore,
the gene for the α′-subunit of cone cGMP-PDE (PDE6C) was a good candidate for screening in autosomal recessive and simplex diseases involving cones such as cone and cone-rod dystrophy. Considering that mutations in a gene expressed in rods are responsible for diseases that significantly affect cones,6,7 it seemed reasonable to analyze a spectrum of retinal degenerative diseases including RP and macular dystrophies for cone cGMP-PDE-α′ mutations as well.

In this study, we have screened patients’ DNAs for mutations in the 22 exons of the cGMP-PDE α′ gene (PDE6C). The PDE6C exons of one subgroup of 91 patients were screened both by single-strand conformation polymorphism electrophoresis (SSCP) and denaturing gradient gel electrophoresis (DGGE), whereas exons of the remaining subgroup of 365 patients were screened only by SSCP.

**METHODS**

**Subjects**

Four hundred fifty-six unrelated patients with retinal degenerative disease were included in this study. Exons in which potential mutant sequence variants were present were screened in control subjects; either in one set of 96 subjects or in a separate set of 30 to 68 subjects. Control subjects self-reported normal vision; their ages and ethnicities were not determined, nor were they examined ophthalmologically. All participants were fully informed of the nature of the investigations, and the research was performed in accordance with institutional guidelines and the Declaration of Helsinki.

All patients were examined clinically with best corrected visual acuity, intraocular pressure, biomicroscopy, direct and indirect ophthalmoscopy, and fundus photography. Visual function testing included standardized Goldmann visual fields and electroretinography. Patients were then categorized into three groups by the mode of inheritance and clinical diagnosis: those with retinal degenerations that have significant effects on cone function as well. Categorized into three groups by the mode of inheritance and clinical diagnosis: those with retinal degenerations that have significant effects on cone function. Those with a predilection for those with retinal degenerations that have significant effects on cone function. Those with retinal degenerations that have significant effects on cone function.

Table 1. Diagnoses of Patients Screened for Mutations in PDE6C

<table>
<thead>
<tr>
<th>Group</th>
<th>Diagnoses</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Cone-rod dystrophy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autosomal recessive</td>
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<td></td>
<td>Simplex</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>Cone dystrophy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simplex</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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</tr>
<tr>
<td></td>
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<td>197</td>
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<tr>
<td>Group 2</td>
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<tr>
<td></td>
<td>Simplex</td>
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<td></td>
<td>Stargardt/fundus flavimaculatus</td>
<td>Recessive</td>
</tr>
<tr>
<td></td>
<td>Simplex</td>
<td>20</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>155</td>
</tr>
<tr>
<td>Group 3</td>
<td>Retinitis pigmentosa</td>
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</tr>
<tr>
<td></td>
<td>Simplex/multiplex and recessive</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>456</td>
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</table>

Included, nor were any patients in whom toxic retinopathy was suspected.

**Screening of Exons by SSCP and DGGE**

DNAs were extracted from blood samples by standard methods and were screened for sequence variants in the PDE6C gene in two laboratories (DBF, EMS). In DBF’s laboratory, exons were screened by both SSCP and DGGE. For SSCP, exons were amplified by polymerase chain reaction (PCR) directly from genomic DNAs with primer pairs that flanked each exon and included intronic sequence on each side. The sequences of the primer pairs are shown in Table 2. The primer pairs were derived from the gene sequences in the GenBank database on the World Wide Web that were deposited by Piriev et al.3 (GenBank Database www2.ncbi.nlm.nih.gov/GenBank Accession numbers U20196 and U20212) The PCR and SSCP protocols have been previously described.4

For DGGE, fragments were amplified directly from genomic DNAs by the same primer pairs used for SSCP, except that one primer of each pair (usually the one with the greater GC content) had a 36-bp “GC clamp” (see Table 2) included before the first 5′ base of the primer; its sequence was kindly provided by John Nakamoto and Neil van Dop (Department of Endocrinology, University of California Los Angeles). The PCR and DGGE protocols have been previously described.4

In EMS’s laboratory, exons were screened by SSCP alone. The PCR protocol was as follows: 5 minutes at 94°C followed by 35 cycles of [94°C for 30 seconds; 50°C, 55°C, 60°C, 65°C for 30 seconds; and 72°C for 30 seconds]. Amplified PCR products were denatured with NaOH, formamide, and heat before they were electrophoresed in sequence-sized 6% nondenaturing acrylamide gels with 5% glycerol. Gels were run at 25 W for approximately 3 hours. After electrophoresis, gels were stained with silver nitrate.10

**Direct Sequencing**

The same primers used to amplify exons by SSCP were used to produce fragments for sequencing. The amplicons were puri-
formed bidirectionally.

Table 2. Primer Pairs Used to Amplify the Exons of the Genes Encoding the α'-Subunit of Cone cGMP-phosphodiesterase

<table>
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<th>Exon</th>
<th>Primer Pair</th>
<th>Exon</th>
<th>Primer Pair</th>
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<tr>
<td>1a</td>
<td>GTGCTCTGAAGGTCGTCCTT</td>
<td>11</td>
<td>*TAGTAAAGGAGATAGGGAATCT</td>
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<td></td>
<td>*AGGAGGTTGGCCAGCTTCTG</td>
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<td>*TGGGACGTGAGGGAGGAGG</td>
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<td>CTGCTTCATAGGCTAAAATCC</td>
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<td>*CTTATGCACCAGAAGCTGAA</td>
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</table>

* For DGGE these primer pairs had the following 5' GC clamp: GCCGCCCGCCGCCGCCCGCCCGCGCCGCCGCCCGCGCGCCCGCGCG.

RESULTS

Exon Screening

Sequence variants were not found in the amplicons of exons 2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 14, 18, and 19 of the PDE6C gene in any of the 456 patients. In the subset of 91 patients screened by both DGGE and SSCP (in DBF's laboratory), several variants were found (Table 3). A heterozygous T–G transversion creating Asp157Glu was detected in 1 patient with AR cone-rod dystrophy (CRD) and 1 of 42 control subjects tested. A second mutation was not found in this patient. A G–A transition in the exon 20 (IVS19+10C→A) and an A–G transition 71 bp upstream of exon 20 (IVS19+71A→G).

There were three additional variants: 1) an A–G transition in the ninth base pair upstream of exon 16 (IVS15–9A→G), 2) a T–C transition in the 29th base pair downstream of exon 17 (IVS17+29T→C), and 3) a G-C transversion in exon 21 creating Lys822Asn. The first was present in 2 patients with AR-CRD and in 0 of 50 control subjects. However, second significant mutations were not found in either of the two patients, and based on the scoring system of Shapiro and Senapathy, this A-G change does not alter the score (efficacy) of this 3' intronic 14-bp splice site. The second variant was found in two patients with AR-CRD and two with simplex CRD and in 0 of 68 control subjects. Significant mutations were not found in any of the four patients, and because splice branch points are approximately 30 to 50 bp upstream of the exon, this downstream variant could not alter an existing branch point or create a new one. The third variant was present in one simplex CRD patient and in 0 of 40 control subjects. However, no second significant mutation was found in the patient, neither parent had disease, and the mother carried the same variant.

In the set of 365 patients whose DNAs were screened only by SSCP (in EMS's laboratory), sequence variants were found in amplicons of exons 8, 10, 20, 21, and 22 (Table 3). The variant in the exon 8 amplicon was the same AL366AL detected in DBF's laboratory, as was IVS9–9C→T; both were also found to be common in patients and control subjects tested in EMS's laboratory. A 6-bp deletion in a stretch of 22 A's starting 96 bp upstream of exon 20 (IVS19–96 to IVS19–75) was found in only 1 patient and 0 of 96 control subjects, but this deletion did not alter or create a branch point (PyNPyPuPy) or create a splice site.11 It was essentially an unchanged sequence.
Three patients and 0 of 96 control subjects had an A–G transition in exon 21 creating Glu834Gly. However in the family of one of the patients, an affected sibling did not carry the variant. The last variant was a 3-bp insertion between the sixth and seventh base pairs after the TAA stop signal in exon 22. This insert was also absent from an affected sibling.

**DISCUSSION**

In our screening for exonic mutations in *PDE6C* in 456 unselected patients with several different forms of retinal disease, we found a number of sequence variants. Five were common polymorphisms present in equal numbers in patients and control subjects, whereas seven others were rare heterozygous sequence variants (Table 3).

The variants were three rare amino acid changes: Asp157Glu, Lys822Asn, and Glu834Gly. None of them could be associated with disease because of the absence of a second variant in autosomal recessive disease, and/or the absence of cosegregation of variant with disease in the probands’ families. The Lys822Asn variant was interesting, because this amino acid residue is in the C-terminal portion of the protein between the highly conserved catalytic site and the highly conserved isoprenylation site in an area with no known functional significance.

There were four additional heterozygous rare sequence variants (Table 3) that were not present in control subjects. None of these could be associated with disease, because second variants could not be found in autosomal recessive cases, and/or they did not cosegregate with disease in the probands’ families, and/or they did not create new or influence existing branch points, and/or they did not influence the sequences around them to create new or influence existing splice sites.

It should be noted that *PDE6C* (or any gene for which negative exon screening results have been obtained) cannot be definitively ruled out as the site for mutations responsible for disease. This is because the SSCP or DGGE techniques may not detect all sequence variants (although only a very small percentage of mutations may remain undetected), mutations may be present in 5′, 3′, or intronic sequences not screened, mutations in this gene may be so rare that not enough patients were screened to detect them, and mutations in this gene may be present only in specific diseases not screened.

Nevertheless, in the patients analyzed, we could find no sequence variants in the gene encoding the α′-subunit of cone cGMP-PDE that could be associated with simplex or autosomal recessive retinal degenerations that predominantly affect cone-mediated function and rod-mediated function or that have a predilection for disease in the macula.

**References**

Inhibition of Vascular Endothelial Cell Morphogenesis in Cultures by Limbal Epithelial Cells

David Hui-Kang Ma, Ray Jui-Fang Tsai, Wing-Keung Chu, Cheng-Heng Kao, and Jan-Kan Chen

PURPOSE. To study the in vitro angiogenic activity of human conjunctival and limbal epithelial cells and conjunctival, limbal, and corneal fibroblasts in a three-cell-type coculture model.

METHODS. Human umbilical vein endothelial cells (EC) were cocultured with epithelial cells, fibroblasts, or epithelial cells and fibroblasts to test their effect on EC morphogenesis. Neutralizing antibodies to some known angiogenic factors were added to the culture to see whether the EC morphogenesis may be blocked by a particular antibody.

RESULTS. Conjunctival and limbal epithelial cells exhibited very little or no stimulatory effect on EC tube formation when examined in an EC–epithelial cell coculture system. In contrast, conjunctival, limbal, and corneal fibroblasts all promoted EC morphogenesis when examined under the same culture conditions. Fibroblast-induced EC morphogenesis was inhibited by addition of anti-vascular endothelial growth factor (VEGF) and/or anti-basic fibroblast growth factor (bFGF) antibodies to the culture medium. In the three-cell-type coculture system consisting of ECs, fibroblasts, and epithelial cells, limbal epithelial cells (but not conjunctival epithelial cells) exhibited a strong inhibitory effect on fibroblast-induced EC tube formation.

CONCLUSIONS. The proangiogenic activity of ocular surface fibroblasts is probably mediated through a paracrine mechanism by VEGF and bFGF. Limbal epithelial cells, but not conjunctival epithelial cells, inhibit fibroblast-stimulated angiogenesis. (Invest Ophthalmol Vis Sci. 1999;40:1822–1828)

Angiogenesis plays important roles in normal tissue functions and in many pathogenic processes. In the ocular surface, vascularization is under strict control, to maintain the transparency and immune privilege of the cornea and the normal differentiation of ocular surface epithelial cells. Derangement of the angiogenic process is believed to have a role in certain external eye diseases. Excessive corneal neovascularization induced by persistent inflammation seen in chemical or thermal burns, infections, autoimmune diseases, such as Stevens–Johnson syndrome and ocular cicatricial pemphigoid, is deleterious to vision. Ischemia of the sclera caused by complications of pterygium excision or severe chemical injuries is also troublesome. Without vascularization, the sclera is subjected to melting and is prone to infection, which again is sight threatening.

Recently, there have been extensive studies concerning corneal angiogenesis; however, most of the studies were focused on the effects of exogenous angiogenic cytokines on corneal neovascularization, and only a few have discussed the mechanism(s) underlying the normal vascularization pattern of the ocular surface. Presumably, in a healthy cornea, antiangiogenic factor(s) is secreted to counteract the angiogenic stimuli from adjacent tissues. When such balance is tipped toward angiogenesis, corneal neovascularization may occur. However, the absence of angiogenic factors is an equally plausible explanation.

The proliferative effect of ocular surface cells (i.e., conjunctival, limbal, and corneal epithelial cells and fibroblasts) on cultured vascular endothelial cells (ECs) has been reported. However, there has been no report concerning their possible effects on EC differentiation. To see whether ocular surface epithelial cells and fibroblasts express differential angiogenic activities in vitro that may be correlated with the vascularization pattern of the ocular surface, we designed a three-cell-type coculture system to examine EC differentiation.
(tube formation) in the presence of various types of ocular surface cells. We found that all ocular surface fibroblasts promote the differentiation of ECs, and such promoting effect was suppressed by limbal epithelial cells (progenitors of corneal epithelial cells). In contrast, under the same culture conditions, conjunctival epithelial cells exerted very little suppressive effect. The promoting effect by fibroblasts is probably mediated by a paracrine mechanism through vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).

**METHODS**

**Tissue Procurement and Cell Culture**

Human eye tissues were procured with a humane method that included proper consent and approval. The study was evaluated and approved by the Medical Ethics and Human Experiment Committee of Chang Gung Memorial Hospital and complied with the ARVO Statement for the Use of Animals in Research and the guidelines of the Declaration of Helsinki in Biomedical Research Involving Human Subjects. Cadaveric tissues were obtained from 22 donors with ages ranging from 2 to 79 years (mean ± SD = 37.3 ± 20.1 years). Conjunctival, limbal, and corneal fibroblasts were grown from tissue explants in HSE medium (25% Ham’s F-12, 75% Dulbecco’s modified Eagle’s medium, 2 ng/ml mouse epithelial growth factor, 1.2 g/l sodium bicarbonate, 0.4 g/l histidine, and 5 × 10⁻⁶ M 2-aminoethanol) supplemented with 10% fetal bovine serum (FBS). Cells were used for experiment between passages two and three.

For isolation of epithelial cells, conjunctival tissues and corneoscleral buttons were treated with 0.5% Dispase II (Boehringer-Mannheim, Indianapolis, IN) in phosphate-buffered saline (PBS) at 37°C for 2 to 3 hours, epithelial cells were then removed and dispersed by gentle scraping and repeated pipetting. Cells were collected by low-speed centrifugation and resuspended in basal medium supplemented with 0.5% dimethyl sulfoxide, 2 ng/ml mouse epithelial growth factor, 1 µg/ml bovine insulin, 0.1 µg/ml cholera toxin, and 5% FBS (SHEM with 5% FBS) and were used immediately in experiments. Limbal and corneal epithelial cells are of the same lineage, but epithelial cells of human central cornea are terminally differentiated cells and did not proliferate in culture.

Vascular ECs were isolated from human umbilical cord vein by type V collagenase (Sigma, St. Louis, MO) digestion. Cells were cultured in MCDB-107 medium (JRH Biosciences, Lenexa, KS) supplemented with 2% FBS, and a fibroblast growth factor–enriched fraction of porcine brain extract at 1 µg/ml. Cells were used below the fifth passage.

**Cocultures**

Type I collagen was extracted from rat tail tendons, as previously described, and was stored as a 3 mg/ml solution in 0.3% acetic acid at 4°C.

Endothelial cells were cocultured with fibroblasts, epithelial cells, or fibroblasts and epithelial cells to test their individual or combined effect on EC tube formation. When included, 8 × 10⁵ conjunctival or limbal epithelial cells were plated on the top of 3 ml type I collagen gel (1 mg/ml) in a six-well tissue culture plate in 2 ml SHEM with 5% FBS. The collagen gel, on which epithelial cells were plated, contained either nothing or were suspended with 8 × 10⁵ human conjunctival, limbal, or corneal fibroblasts. To make fibroblast-suspended collagen gel, 1 ml collagen solution (3 mg/ml) was mixed with 1 ml 2 × HSE medium and was neutralized with appropriate amount of 5 N NaOH. The mixture was then mixed with 1 ml 1 × HSE medium containing 8 × 10⁵ fibroblasts. Three milliliters of collagen-cell mixture was poured per well in a six-well plate and incubated at 37°C, where it gelled in a few minutes.

The collagen gels contained with or without fibroblasts and with or without epithelial cell overlying were cultured for 24 hours, and the medium was replaced with the same medium. The ECs were then harvested from subconfluent monolayer cultures and suspended in collagen solution at 5 × 10⁵ cells/ml, as described, and 1 ml was poured per Falcon cell culture insert (Becton Dickinson, Franklin Lakes, NJ). After the collagen–EC mixture had gelled, the inserts were placed to allow the interactions of EC with fibroblasts, epithelial cells, fibroblasts and epithelial cells, or acellular collagen gel. Cultures were maintained for 3 days and were fixed with 4% formaldehyde in PBS. Endothelial cell tube formation was then quantified by a computerized image analysis system connected to a phase-contrast microscope (IMT-2; Olympus, Lake Success, NY). At least three duplicates were performed in each experiment. Tube length in five random fields per insert was computed and was expressed as millimeters per well. The EC gels and the epithelial cell–fibroblast gels were then embedded in paraffin, and were processed for hematoxylin-eosin (HE) staining. In addition, the latter were also stained with periodic acid–Shiff (PAS) and Alcian blue to detect conjunctiva-specific goblet cells.

**Effect of Neutralizing Antibodies to VEGF and bFGF on Fibroblast-Induced Capillary Tube Formation**

To explore the potential role of major angiogenic cytokines in the regulation of fibroblast-induced EC differentiation, 2 × 10⁵ limbal fibroblast in 1 ml/well type I collagen solution (1 mg/ml) was poured into a 24-well culture plate. After incubation at 37°C for 24 hours, 2.5 × 10⁵ EC in 0.5 ml collagen solution was overlaid on the fibroblast-suspended collagen gel. After the EC–collagen layer gelled, 0.5 ml MCDB-107 medium with 2% FBS was added per well. Neutralizing antibodies to VEGF (monoclonal, ranging from 0.1 to 1.0 µg/ml) and/or bFGF (polyclonal, ranging from 1.0 to 10 µg/ml, R&D Systems, Minneapolis, MN) were added. Irrelevant rabbit IgG (Sigma) was added at the same concentration range as control. Cells were cultured for 3 days, and the total capillary tube length per well was compared and expressed as a percentage of control.

**Statistics**

Total capillary tube length between two groups were compared using two-sample unpaired t-test or the Mann-Whitney test when appropriate. All P determinations were two sided and were considered significant when P < 0.05.

**RESULTS**

After coculturing for 3 days, the fibroblast-suspended collagen gels usually contracted to a diameter half the original size. The overlaying epithelial cells formed a confluent cell sheet. Histologic examination showed that the conjunctival epithelial cell layer formed on fibroblast-dispersed gel was mostly one cell in
depth with occasional patches that were two cells thick, and the limbal epithelial cell layer was a uniform monolayer of cells (not shown). The conjunctival epithelial cell layer contained PAS- and Alcian blue–positive cells, indicating the presence of glycoconjugate-expressing cells. In contrast, limbal epithelial cell layers were devoid of PAS- and Alcian blue–positive cells (not shown). This observation is similar to previous reports of cocultures of fibroblast with conjunctival epithelial cells or limbal epithelial cells under the submerged condition9,12 and is consistent with the in vivo observation that conjunctival and limbal epithelial cells belong to separate lineages.13

When cocultured with acellular collagen gel, the ECs remained rounded, and there was no sign of EC morphogenesis. When cocultured with either conjunctival epithelial cells (Fig. 1A) or limbal epithelial cells (Fig. 2A), most of the ECs still remained rounded; however, a few reorganized ECs were observed. On coculture with either conjunctival fibroblasts (Fig. 1B), limbal fibroblasts (Fig. 2B), or corneal fibroblasts (Fig. 3A), prominent EC morphogenesis was observed. The ECs migrated and organized into capillary-like networks.

To see whether the paracrine interactions between epithelial cells and fibroblasts have any influence on EC morphogenesis, conjunctival and limbal epithelial cells were cocultured with corresponding fibroblasts. In such three-cell-type cultures, the capillary tube formation by the ECs was reduced compared with those cocultured with fibroblasts alone. In three-cell-type cultures containing conjunctival epithelial cells and conjunctival fibroblast (Fig. 1C) or conjunctival epithelial cells and corneal fibroblasts (Fig. 3B), the total length of the capillary network was slightly decreased. Figure 1 shows that the total capillary tube length in EC–conjunctival fibroblast coculture was 2974.0 ± 765.6 (mean ± SD) mm, and tube length in EC–conjunctival epithelial cell–conjunctival fibroblast coculture was 2757.8 ± 876.7 mm. (7.3% decrease; P = 0.585). Figure 3 shows that the total capillary tube length in the EC–corneal fibroblast cell coculture was 3846.0 ± 279.3 mm, and tube length in EC–conjunctival epithelial cell–corneal fibroblast coculture was 3535.9 ± 422.3 mm (8.1% decrease; P = 0.302). As is shown in Figure 1C, some cords were formed by clusters of EC instead of by well-aligned, elongated cells similar to those seen in conjunctival fibroblast-induced cultures.

When conjunctival epithelial cells were replaced by limbal epithelial cells in the three-cell-type cocultures, EC morphogenesis was reduced significantly. As is shown in Figures 2B, 2C, and 2D, total capillary tube length in EC–limbal fibroblast coculture was 4789.9 ± 1104.2 mm, and that in EC–limbal epithelial cell–limbal fibroblast coculture was 2566.7 ± 566.3 mm (46.4% decrease; P < 0.001). A similar effect was observed when limbal fibroblasts were replaced by corneal fibroblasts.
There was a 44.0% (P < 0.001) reduction in the total capillary tube length when limbal epithelial cells were included in the EC–corneal fibroblast coculture system (Fig. 3). In EC-limbal epithelial cell coculture, ECs stayed rounded or were slightly elongated, but did not reorganize.

The results clearly showed that conjunctival, limbal, and corneal fibroblasts all induced prominent capillary tube formation. It would be interesting, therefore, to see what soluble factor(s) may be involved. Various neutralizing antibodies to various selective EC mitogens were tested for their ability to block fibroblast-induced EC morphogenesis. Although irrelevant antibody had no effect on EC morphogenesis, we found that neutralizing antibody to VEGF at 0.1 and 1.0 μg/ml inhibited capillary tube formation by 62.5% and 86.4%, respectively. Moreover, neutralizing antibody to bFGF was also effective; at 1.0 and 10 μg/ml, it exerted, respectively, a 46.7% and 60.1% inhibition. The inhibitory effect of both antibodies appeared to be additive. Simultaneous presence of both antibodies at appropriate concentrations almost completely inhibited fibroblast-induced capillary tube formation.

DISCUSSION

In vitro models of angiogenesis are usually incomplete, because only some early cellular events of the processes are investigated. However, it offers a simplified system to analyze the roles of cell–cell and cell–matrix interactions, and to avoid confounding factors such as inflammatory cells and factors from circulation. It is generally believed that corneal neovascularization is a manifestation of inflammation and that infiltrating leukocytes play a major role. However, an earlier experiment showed that even in leukopenic mice, neovascularization still occurs in response to chemical injury, although to a much lesser degree. It therefore raises the possibility that cells other than leukocytes or noncellular constituents of inflammation may also contribute to the angiogenic signals. Fibroblasts and epithelial cells from other tissues have been shown to express VEGF and to induce capillary formation by ECs. It is, therefore, reasonable to hypothesize that ocular surface epithelial cells and fibroblasts also express angiogenic activities and participate in the regulation of ocular surface vascularization.

It has been suggested that active cytokine cross talk between ocular surface epithelium and fibroblasts is present and may have a regulatory role in important cellular processes such as epithelial differentiation or corneal wound healing and, as proposed in this article, the angiogenic phenotype of the ocular surface. It is difficult, if not impossible, to analyze the effects of cytokine cross talk on cell behavior in the in vivo system. Thus, development of culture systems with closer
mimicry of the in vivo tissue may be necessary if this problem is to be approached. In this regard, we recently reported the fabrication of in vitro human conjunctival equivalents from conjunctival epithelial cells, fibroblasts, and type I collagen and clearly showed the regulatory effect of stromal fibroblasts on epithelial cell development and differentiation. In this study, we extended the coculture system to contain vascular ECs and examined the effect of epithelial–fibroblast interactions on EC differentiation.

Studies of the proliferative effect (on ECs) and cytokine production by others all indicate that conjunctival epithelial cells are angiogenic, whereas limbal and corneal epithelial cells are antiangiogenic. However, the role of stromal fibroblasts and epithelial–fibroblast interactions is less defined. In this study, we clearly showed that conjunctival, limbal, and corneal fibroblasts all promoted prominent capillary tube formation by ECs, indicating the expression of proangiogenic activity by these cells. The observation is supported by enzyme-linked immunosorbent assay and reverse transcription–polymerase chain reaction results (not shown) showing that all these cells express comparable amounts of VEGF protein and mRNA in culture. Although it has been reported that bFGF mRNA was differentially expressed among ocular surface fibroblasts and that interleukin-1β upregulates bFGF more in limbal than in corneal fibroblasts, the present study suggests that different ocular surface fibroblasts express comparable angiogenic activity in vitro. In our study, fibroblast-suspended collagen gels without overlying epithelial cells may be viewed as a mimicry of the in vivo fibroblast activation during epithelial defect (wounding). With reepithelialization, fibroblast activity is then downregulated. Compared with conjunctival epithelial cells, limbal epithelial cells significantly downregulated corneal fibroblast–induced EC morphogenesis, indicating the antiangiogenic property of the limbal epithelial cell.

Thus, ocular surface fibroblasts may not be a major determinant in the regional difference of ocular surface vascularization. Moreover, in normal in vivo conditions, fibroblasts are quiescent and exhibit very little cytokine or extracellular matrix production. It has been suggested that ocular surface cells may shift to an angiogenic phenotype in response to external stimuli such as hypoxia or inflammation and may thus have implications in the pathogenesis of pterygium recurrence or pannus formation. In addition, fibroblasts may produce chemokines such as interleukin-8 in response to bacterial prod-
ucts. Interleukin-8 has been shown to induce corneal neovascularization and regulates inflammatory reactions.

The in vitro observations of antiproliferative effect on ECs, and the inhibition of EC morphogenesis by limbal epithelial cells in our study may not be readily interpreted to have antiangiogenic activity in vivo. However, some clinical observations are supportive of this notion. The stem cells of corneal epithelium reside in the basal layer of limbal epithelium. Clinical observations show that after severe chemical injuries, infection, or autoimmune diseases with significant loss of limbal stem cells, the corneal surface is repopulated with conjunctival epithelium accompanied with chronic inflammation and prominent fibrovascular ingrowth. Current treatment for this vision-threatening dilemma is to perform limbal transplantation. By providing healthy stem cells of corneal epithelium from the contralateral eye or cadaveric eyes, the surgery not only restores corneal epithelial phenotype, but also effectively reduces corneal neovascularization. Because limbal fibroblasts are proangiogenic in phenotype, it is thus possible that transplanted limbuscorneal epithelial cells exert antiangiogenic activity.

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References

Visual Function Correlates with Nerve Fiber Layer Thickness in Eyes Affected by Ocular Hypertension

Vincenzo Parisi,1,2,3 Gianluca Manni,1,2 Stefano A. Gandolfi,2 Marco Centofanti,1,2,3 Gaspare Colacino,1,2 and Massimo G. Bucci1,2

PURPOSE. To test whether the high variability observed when measuring pattern electroretinogram (PERG), visual evoked potentials (VEP), and spatial contrast sensitivity (SCS) in eyes with ocular hypertension is associated with variation in nerve fiber layer thickness, as measured by optical coherence tomography (OCT).

METHODS. The study involved 32 untreated eyes (32 patients; age range, 29–64 years) showing a normal white-on-white 24/2 Humphrey (San Leandro, CA) perimetry, IOP between 23 and 28 mm Hg, best corrected acuity of better than 28 mm Hg (average of the two highest readings of the previous 3 years). Enrollment was conducted according to the following inclusion criteria: IOP more than 23 mm Hg and less than 28 mm Hg (average of the two highest readings of the daily curve, from 8:00 AM to 6:00 PM, six independent readings, one every 2 hours); normal automatic full threshold perimetry (24/2 Humphrey, mean defect, corrected pattern standard deviation and glucoma hemifield test within the normal range of the database of the Humphrey [San Leandro, CA]) software; fixation losses, false-positive rate and false-negative

RESULTS. The 95% confidence limits of the electrophysiological data were: PERG P50 latency, 59.3 to 65 msec; PERG P50 to N95 amplitude, 0.74 to 1.15 μmV; VEP P100 latency, 113 to 118 msec; VEP N75 to P100 amplitude, 3.81 to 4.90 μmV. The 360° nerve fiber layer thickness overall (NFLO) ranged between 113 and 169 μm (145 ± 16 μm; mean ± SD) and significantly correlated with PERG P50 to N95 amplitude (r = 0.518; P = 0.002), PERG P50 latency (r = −0.470; P = 0.007), VEP N75 to P100 amplitude (r = 0.460; P = 0.008), VEP P100 latency (r = −0.422; P = 0.016) and SCS at 3 cyc/deg (r = −0.358; P = 0.358).

CONCLUSIONS. The variability of PERG, VEP, and SCS testing observed in eyes with ocular hypertension is associated with differences in NFL thickness (the thinner the layer, the worse the visual function). (Invest Ophtalmol Vis Sci. 1999;40:1828–1833)

Psychophysical1 and electrophysiological2,3 experiments have described impaired visual function in human eyes having high intraocular pressure (IOP) but showing a normal visual field tested by white-on-white computer-assisted static perimetry. However, a significant overlap exists between normal subjects and those with ocular hypertension.2,3

Optical coherence tomography (OCT), a recently developed technique, allows in vivo scanning of the retinal layers. The device is based on the interferometry principle, with a superluminescent diode used as a source. The resolution limits are approximately 10 μm.4 Measurements of retinal thickness are obtained automatically by means of a computer algorithm that searches for the characteristic changes in reflectivity observed at the superficial and deep retinal boundaries.4 Experiments performed on glaucomatous eyes have extensively shown topographical correlation between visual field defects and localized or diffused thinning of the nerve fiber layers (NFLs).5

We applied OCT to eyes with ocular hypertension and no visual field defects. The data on the NFLs were then correlated with both electrophysiological (pattern electroretinogram [PERG] and visual evoked potentials [VEPs]) and psychophysical (spatial contrast sensitivity [SCS]) parameters. Thus, we tested whether the reported variability is associated with interindividual variation in NFL thickness.

METHODS

Thirty-two eyes of 32 consecutive patients (age range, 29–64 years, mean, 48 ± 9 years) affected by ocular hypertension were recruited. Each patient had to be experienced with automatic perimetry (at least six reliable examinations within the previous 3 years). Enrollment was conducted according to the following inclusion criteria: IOP more than 23 mm Hg and less than 28 mm Hg (average of the two highest readings of the daily curve, from 8:00 AM to 6:00 PM, six independent readings, one every 2 hours); normal automatic full threshold perimetry (24/2 Humphrey, mean defect, corrected pattern standard deviation and glucoma hemifield test within the normal range of the database of the Humphrey [San Leandro, CA]) software; fixation losses, false-positive rate and false-negative
rate each <20%); best corrected visual acuity 20/20 or better; none of the following papillary signs on conventional color stereo slides: rim notches, peripapillary splinter hemorrhages, increased vertical-to-horizontal cup-to-disc ratio, cup-to-disc asymmetry between the two eyes less than 0.2; mean refractive error (when present) between −0.50 and +0.50 spherical equivalent; no previous history of diabetes, optic neuritis, or any disease involving the anterior visual pathways; and pupil diameter 3 mm or more.

On recruitment, each patient provided informed consent to the procedures. The research followed the tenets of the Declaration of Helsinki.

Each eye underwent the following procedures: SCS testing and OCT were performed on the same day; PERG and VEP recordings were performed 1 week later.

**OCT Examination**

OCT (Humphrey) was performed using a fiber-optic delivery system coupled with slit lamp biomicroscope. This system provides the operator with a video camera view of the scanning probe beam on the fundus and an OCT image acquired in real time on a computer monitor.

After dilation with 1% tropicamide, each eye was scanned three times using a 3.4-mm circle (1.7-mm radius). Near-infrared light (840-nm wavelength) was used. Throughout scanning, the patient kept the eyes fixed on an internal target provided by the equipment. The measurements were obtained from three nonconsecutive scans (i.e., the patient was allowed to rest for a few seconds before repositioning to proceed to the next scan).

The OCT software has an automated computer algorithm that identifies the anterior and posterior border of the retina, making it possible to calculate NFL and total retinal thickness overall by quadrant and by clock hour.

In the assessed eyes, we considered the average of the values obtained by three different measurements in each quadrant: superior (NFLS), inferior (NFLI), nasal (NFLN), and temporal (NFLT); the overall data obtained in all quadrants (12 values averaged) were identified as NFL overall (NFLO).

**Contrast Sensitivity Examination**

Foveal SCS was tested by using a commercial available chart (CSV1000, Vector Vision, Daytona, OH). The CSV1000 provides a fluorescent luminance source that retroilluminates a translucent chart. The instrument houses a series of photocells that automatically monitor and calibrate the instrument light level to 85 candelas (cd/m²) ± 0.1 log unit. At the testing distance of 8 feet, the translucent chart presents four spatial frequencies, each on a separate row of the test: 3, 6, 12, and 18 cycles/deg. Sensitivity levels at each frequency range from 0.7 to 0.17 to 1.55 (18 cycles/deg) log units.

The procedure described by Pomerance and Evans was followed. Sensitivity threshold was measured two times, allowing only a few seconds between measurements. Only the second measurements were considered for analysis. The test-retest variability was consistent with that reported previously.

**Electrophysiological Examination**

Simultaneous recordings of transient VEPs and PERGs were assessed using a method previously published. The subjects under examination were seated in a semidark, acoustically isolated room in front of a display that was surrounded by a uniform field of luminance of 5 cd/m². The subjects were informed of the type of examination and its diagnostic uses. Before the experiment, each subject was adapted to the ambient room light for 10 minutes, and the pupil diameter was approximately 5 mm. Miotic or mydriatic drugs were not used.

The visual stimuli were checkerboard patterns (contrast 70%, mean luminance 110 cd/m²) generated on a TV monitor and reversed in contrast at the rate of two reversals per second. At the viewing distance of 114 cm the check edges subtended 15 minutes of visual angle and the screen of the monitor subtended 12.5°. The refraction of all subjects was corrected for the viewing distance. We used 15 minutes of visual angle, because this smaller size is considered optimal to stimulate the fovea in pattern electroretinography as well. The stimulation was monocular, with occlusion of the contralateral eye.

**PERG Recordings**

This bioelectrical signal was recorded by a small Ag/AgCl skin electrode placed over the lower eyelid. PERGs were derived bipolarly between the stimulated eye (active electrode) and the patched eye (reference electrode). The ground electrode was in Fpz. The interelectrode resistance was lower than 3 kΩ.

The signal was amplified (gain 50,000), filtered (band-pass, 1-30Hz), and averaged with automatic rejection of artifacts (200 events free from artifacts were averaged for every trial) by BM 6000 (Biomedica Mangoni, Pisa, Italy). The analysis time was 250 msec.

The transient PERG response is characterized by a series of waves with three subsequent peaks, of negative, then positive, then negative polarity. In normal subjects when the conditions of our experiment are used, these peaks have the following mean latencies: 35, 50, and 95 msec.

**VEP Recordings**

Cup-shaped Ag/AgCl electrodes were fixed with collodion in the following positions: active electrode at Oz, reference electrode at Fpz, ground on the left arm. The interelectrode resistance was kept below 3 kΩ. The bioelectric signal was amplified (gain 20,000), filtered (band-pass, 1-100 Hz), and averaged (200 events free from artifacts were averaged for every trial) by BM 6000. The analysis time was 250 msec.

The transient VEP response is characterized by a series of waves with three sequential peaks, of negative, then positive, then negative polarity. In normal subjects and in the conditions of our experiment, these peaks have the following mean latencies: 75, 100, and 145 msec.

In the recording session, simultaneous PERGs and VEPs were recorded with at least two replications, and the resultant waveforms were superimposed to check the repeatability of the results. We accepted PERG and VEP signals with signal-to-noise ratio more than 2. The noise was measured by recording the bioelectrical signals (200 averaged events) while the monitor was screened by a cardboard and a noise less than 0.1 μV (mean 0.085 μV) was observed in all subjects tested.

For all PERGs and VEPs the peak latency and the peak amplitude of each of the averaged waves were measured directly on the displayed records by means of a pair of cursors. By comparing the VEP peak latency (P100) and the PERG peak latency (P50), it is possible to have an index of postretinal...
neural conduction. We call the difference between VEP P100 latency and PERG P50 latency retinocortical time (RCT).\(^5\)

**Statistics**

Spearman rank and linear regression analysis (Pearson’s test) were adopted to evaluate the correlations among SCS, PERG, and VEP parameters and the NFL thickness determined by OCT. Data are expressed as mean ± SD.

**RESULTS**

The data collected from each patient are displayed in Table 1. The electrophysiological recordings produced the following results (95% confidence limits): PERG P50 latency, 59.3 to 63 msec; PERG P50 to N95 amplitude, 0.74 to 1.15 μV; VEP P100 latency, 113 to 118 msec; VEP N75 to P 100 amplitude, 3.81 to 4.90 μV.

The variability of the electrophysiological data were examined as a function of NFL thickness. As shown in Figure 1A, 1B, 1C, and 1D, the experimental points can be fit by linear regressions whose level of significance is reported in Table 2A.

The thickness of the NFL, measured across the 360° section along the optic disc (NFLO), ranged between 113 and 169 μm (145 ± 16 μm). The NFL thickness values of each patient showed a significant correlation with PERG P50 to N95 amplitude (r: 0.518; \(P = 0.002\)), PERG P50 latency (r: -0.470; \(P = 0.007\)), VEP N75 to P 100 amplitude (r: 0.460; \(P = 0.008\)), and VEP P100 latency (r: -0.422; \(P = 0.016\)). Because the software of the OCT allows the dissection of four quadrants from the 360° scan, we tested each sector for possible correlation with the electrophysiological data. As shown in Table 2A, PERG and VEP parameters correlated well with the NFL data obtained by OCT in the NFLS, NFLT, NFLI, and temporal (NFLT, including the papillomacular bundle) sector.

NFLO was correlated with SCS threshold measured at 3 cyc/deg (r: -0.358; \(P = 0.044\)); NLF and NFLT were also significantly (P < 0.01) correlated with SCS threshold measured at 3 cyc/deg. No apparent correlation was observed between SCS measured at 6, 12, and 18 cyc/deg and the OCT data. The levels of significance for each correlation are detailed in Table 2B. Age and IOP showed no correlation with OCT-measured NFL thickness (see Table 2A for details).

### Table 1. Observed Characteristics in Patients with Ocular Hypertension

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* Average of the two highest IOP values in the daily curve in millimeters of mercury.
† All NFL data are in micrometers.
‡ PERG P50 peak latency in milliseconds.
§ PERG P50 to N95 amplitude in microvolts.
¶ VEP N75 to P100 amplitude in microvolts.
# VEP P100 peak latency in milliseconds.
DISCUSSION

In this study, we examined eyes with ocular hypertension and no evidence of field damage, and we measured NFL thickness (OCT), PERGs, VEPs, and SCS.

When considering eyes affected by high IOP and no sign of field damage, the actual risk for a conversion to glaucoma is not presently known. Because our study was carried out cross-sectionally, no information is available to determine which eye would eventually have a field defect.

The NFL thickness range obtained by OCT analysis in our study eyes is consistent with that previously reported in a similar population. Most of the eyes showed OCT values well above the suggested lower limits for normal subjects (125–135 μm). The scattering of the electrophysiological data (see Fig. 1) is consistent with that previously observed by investigators in several studies of ocular hypertension. When plotting the data as a function of the OCT values, data can be fit by linear regression. A similar phenomenon is observed for the 3-cyc/deg threshold of SCS. These correlations, albeit moderate, show a strong significance. This result suggests that eyes having a thinner ganglion cell layer, produce smaller electrophysiological (both retinal [PERG]) and cortical [VEP]) and psychophysical responses.

Several psychophysical studies have found that contrast sensitivity to low spatial frequency patterns is impaired in patients affected by glaucoma and other diseases, leading to a pathologic contrast sensitivity, appears to be in the low-frequency region. As pointed out by Bodis-Wollner, low spatial frequency refers to patterns with a frequency near, but lower than, the peak of the human foveal contrast sensitivity curve. Three cycles per degree fits this model. Should a lower signal from the OCT (i.e., a thinner NFL) represent a sign of early ganglion cell loss, 3 cyc/deg would be the most likely affected frequency among those tested by the CSV1000 chart. Not surprisingly, then, 3 cyc/deg was the only frequency with a threshold that correlated well with the NFL thickness in our series of hypertensive eyes (see Table 2B).

Studies performed in animal models have shown that the PERG reflects the bioelectrical activity of the innermost retinal layers (the ganglion cells and their fibers). The existence of similar evidence in humans is still controversial. However, if the PERG generators in humans are in the innermost retinal layers, then our data show an intriguing relationship between an electrophysiological bioelectrical response (PERG) and its supposed anatomic counterpart (i.e., the innermost retina) in humans.

Age may have been a potentially confounding factor in the analysis of the data. However, as shown in Table 2A, there was no correlation between age and NFL thickness measured in our cohort of patients. Therefore, we believe that this potential source of bias was, in our study, negligible.

**FIGURE 1.** NFL O (dashed line) and NFLT (solid line) plotted versus PERG P50 latency (A), PERG P50 to N95 amplitude (B), VEP P100 latency (C), and VEP N75 to P100 amplitude (D). Statistical analysis is reported in Table 2A.
We also observed a strong correlation between VEP electrophysiology and OCT. The cortical evoked response (VEP) is derived from retinal activity together with neural conduction along the visual pathways. When simultaneously recording PERG and VEP, an index of neural conduction (the so-called RCT) can be obtained. In patients with glaucoma, a delay in postretinal visual pathways (i.e., increased RCT) has only been observed once an eye has an actual field defect. Eyes with ocular hypertension, but without field defects, have shown normal postretinal neural conduction. Comparable data may be obtained from the recordings obtained in our cohort of patients. As shown in Table 1, RCT calculated in our study (54.8 ± 4.55 msec) can be superimposed on that previously reported in a cohort of patients with ocular hypertension (54.3 ± 4.12 msec). We can, therefore, assume that the postretinal neural conduction in our cohort of patients was within the normal range. Therefore, the variability of the VEP response, observed in our patients, would be best explained by differences in the retinal anatomy only. The correlation between NFL thickness and the VEP responses (see Table 2A) suggests that, in our cohort of ocular hypertensive eyes, the neural conduction in the visual pathways was dominated by the retinal component (i.e., the thinner the layers, the worse the conduction).

In conclusion, the data collected in our study show that the variability of the PERG, VEP and SCS threshold observed in eyes with ocular hypertension, is correlated with interindividual variation in NFL thickness (the thinner the layer, the worse the visual function). In human eyes, the NFL thickness measured by OCT is correlated with electrophysiological responses assumed to be originating in the innermost retinal layers.

However, we want to emphasize that a significant correlation between two factors does not imply causality. Other as yet unrecognized factors may contribute to the observed variability of functional parameters in eyes with ocular hypertension.

References

Isoproterenol, Forskolin, and cAMP-Induced Nitric Oxide Production in Pig Ciliary Processes

Rong Liu, Josef Flammer, and Ivan O. Haefliger

PURPOSE. To investigate whether isoproterenol and forskolin, two adenylyl cyclase activators, or 8-bromo-cAMP, an adenosine 3',5'-cyclic monophosphate (cAMP) analog, increase nitric oxide (NO) production in isolated porcine ciliary processes.

METHODS. Nitrite (an NO metabolite) was measured (Griess reaction) before and 2 hours after exposure to 0.1 to 100 μM isoproterenol (a β-adrenoreceptor agonist, 0.01 to 100 μM forskolin, or 0.1 to 1000 μM 8-bromo-cAMP. Some experiments were conducted in the presence of 0.5 mM Nω-nitro-l-arginine methyl ester (l-NAME; a nitric oxide synthase [NOS] inhibitor), 10 μM propranolol (a β-adrenoreceptor antagonist), or 1 μM KT 5720 (a cAMP-dependent protein kinase inhibitor). cAMP production was also measured (by immunoassay).

RESULTS. Nitrite production was increased by isoproterenol (maximum, 10 μM: 164%; P < 0.001), forskolin (maximum, 10 μM: 254%; P < 0.001), and 8-bromo-cAMP (maximum, 100 μM: 184%; P < 0.001), an effect prevented by l-NAME (P < 0.05–0.001). Propranolol inhibited only isoproterenol-induced (10 μM) nitrite production (P < 0.05), whereas KT 5720 (P < 0.05) inhibited isoproterenol- (10 μM) and 8-bromo-cAMP-induced (10 μM) nitrite production. Furthermore, CAMP production evoked by isoproterenol (10 μM, P < 0.05) but not by forskolin (10 μM, P < 0.001) was inhibited by propranolol (P < 0.05).

CONCLUSIONS. In isolated porcine ciliary processes, drugs activating adenyl cyclase or mimicking cAMP increase the production of NO by a mechanism that appears to involve both a CAMP-dependent protein kinase and NOS.

In the eye, the primary site of aqueous humor formation is the epithelium of the ciliary processes. By a mechanism that still is unclear, drugs that decrease intracellular concentration of cyclic 3',5'-adenosine monophosphate (cAMP), such as β-adrenergic receptor antagonists (β-blockers), also inhibit aqueous humor formation.1

Nitric oxide (NO) is a cellular mediator that can be produced by the enzyme nitric oxide synthase (NOS) from the amino acid l-arginine.2 Nitric oxide has a short half-life and is rapidly transformed in more stable compounds, such as nitrite (NO2). In the kidney, the respiratory airway, and the colon, NO can modulate transepithelial ionic and/or fluid transport.3–5

The presence of an NOS activity6 that can be modulated by β-adrenergic agents7 has recently been reported in porcine ciliary processes, raising the possibility that NO could be associated with the process of aqueous humor formation. This study investigated whether in isolated porcine ciliary processes NO production is increased by drugs that either activate adenylyl cyclase, such as isoproterenol (a β-adrenergic receptor agonist) and forskolin (an adenylyl cyclase activator), or mimic cAMP, such as 8-bromo-cAMP (a stable lipophylic cAMP analog).

MATERIALS AND METHODS

Tissue Preparation

In adherence to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research, porcine eyes (one eye/animal) were obtained from an abattoir immediately after animals were killed and were transported to the laboratory in ice-cold Krebs-Ringer's physiologic solution. Within 3 hours after death of the animal, the ciliary processes were dissected in cold Krebs-Ringer's solution. Tissues were then either stored at −70°C until nitrite measurements were conducted or used right away for cAMP determination.

Nitrite Measurements

Tissues were thawed at room temperature and washed with Hanks’ balanced salt solution (HBSS). Ciliary processes were placed in a 24-well plate (ciliary processes from one eye/well), covered with 700 μl HBSS–l-arginine medium (HBSS supplemented with 10 μM L-arginine), and incubated in a humidified incubator at 37°C. After 30 minutes, 100 μl medium was taken from each well for the first nitrite measurement. Tissues were then ex-
posed for 2 hours to different drugs (agonists and/or antagonists) before another 100-μl sample was collected in each well for a second nitrite measurement. For each experiment, quiescent controls were run in parallel in which tissues were not exposed to tested drugs. Nitrite production was defined as the amount of nitrite measured between the first and second measurements.

Nitrite was assessed by Griess reaction. In brief, samples were centrifuged at 1000g for 15 minutes. From each sample, 45 μl supernatant was mixed with 45 μl Griess reagent (0.75% sulfanilamide in 5% phosphoric acid and 0.075% N-(1-naphthyl)ethylenediamine dihydrochloride in double-distilled water) and incubated at room temperature for 10 minutes. Optical density was then measured on a microplate reader at 540 nm. Nitrite concentrations were determined by comparisons with a standard sodium nitrite curve.

**cAMP Measurements**

In a 24-well plate, freshly dissected tissues (ciliary processes from one eye/well) were incubated in HBSS for 60 minutes at 37°C before exposure for 30 minutes to 0.5 mM isobutyl-methylxanthine, an inhibitor of cyclic nucleotide phosphodiesterase to prevent breakdown of accumulated nucleotides. Tissues were then further incubated for 30 minutes with the nonselective β-adrenergic receptor antagonist propranolol (10 μM) before exposure for 10 minutes to isoproterenol (10 μM) or forskolin (10 μM). Similar experiments were also run in parallel without propranolol. At the end of the experimental protocol, tissues were rapidly frozen in liquid nitrogen and stored at −70°C until assayed for cAMP determination.

For cAMP measurement, each sample was homogenized at 4°C in the presence of 0.5 ml ice-cold 6% trichloroacetic acid (TCA) before they were centrifuged at 2000g for 20 minutes at 4°C. The supernatant was removed, placed in a test tube, and extracted four times with water-saturated ethyl ether. The extracted supernatant was further evaporated by a centrifuged vacuum pump at 40°C, and the cAMP content measured with a commercial enzyme immunoassay kit (Amersham, Amersham, UK). Experiments were repeated three times in duplicate. The amount of protein in each well was measured by dissolving the pellets in 1 ml of 0.1 N NaOH and assayed for protein concentration using a commercially available kit (Bio-Rad, Glattbrugg, Switzerland). The amount of cAMP was normalized to the amount of protein in each well.

**Drugs**

L-Arginine, 8-bromo-cAMP, dimethyl sulfoxide (DMSO), forskolin, isobutyl-methylxanthine, (-)-isoproterenol, KT 5720, KT 5823, N⁵-nitro-L-arginine methyl ester (L-NAME), N⁵-nitro-D-arginine methyl ester (D-NAME), DL-propranolol, sodium nitrite, and TCA were purchased from Sigma (Buchs, Switzerland). Griess reagent was obtained from Merck (Darmstadt, Germany). HBSS was purchased from Gibco (Basel, Switzerland). All drugs were made up fresh the day of the experiment. The drugs were dissolved in HBSS except for forskolin, KT 5720, and KT 5823, which were dissolved in DMSO and isoproterenol for which ethanol was used. The final maximal concentration in a well for DMSO and ethanol was 0.1% and 0.01%, respectively.

**Statistical Analysis**

Nitrite production (the difference between the first and second nitrite measurements) was either expressed in micromolar per milligram of tissue or in the percentage of the mean nitrite production of quiescent controls (% of control) run in parallel with each experiment. Data of cAMP measurements were expressed in picomoles per milligram protein. Results are shown as mean ± SEM with n corresponding to the number of eyes assessed (ciliary processes from one eye per animal per well). Statistical comparisons were conducted using either an unpaired Student’s t-test or a one-way analysis of variance followed by Bonferroni’s multiple-comparison test with P < 0.05 considered to be significant.

**RESULTS**

**Basal Nitrite Production**

In quiescent isolated ciliary processes, the basal production of nitrite (1.3 ± 0.2 μM/mg tissue; n = 20) was significantly (P < 0.05) inhibited by 0.5 mM L-NAME, an inhibitor of NO formation (0.7 ± 0.2 μM/mg tissue; n = 20). These results indicate the existence of basal NOS activity in isolated porcine ciliary processes.

**cAMP-Induced Nitrite Production**

In a concentration-dependent manner, the basal production of nitrate was significantly increased by the β-adrenergic receptor agonist isoproterenol (0.1–100 μM), the adenylylclase activator forskolin (0.01–100 μM), or the stable and lipophilic cAMP analog 8-bromo-cAMP (1–1000 μM). The maximal increase in nitrite production was observed at a concentration of 10 μM for isoproterenol (P < 0.001), 10 μM for forskolin (P < 0.001), and 100 μM for 8-bromo-cAMP (P < 0.001). These results indicate that in isolated porcine ciliary processes the production of nitrate can be enhanced by drugs known to activate adenylylclase (isoproterenol, forskolin) or to mimic cAMP (8-bromo-cAMP; Fig. 1).

**cAMP-Induced Nitrite Production and L-NAME**

The increase in nitrite production evoked by isoproterenol (10 μM) was significantly (P < 0.01) blunted by the inhibitor of NO formation L-NAME (0.5 mM), but not by D-NAME (0.5 mM), the dextrorotatory nonmetabolizable isomer of L-NAME. Also, the nitrite production induced by forskolin (1 μM; P < 0.001) or 8-bromo-cAMP (10 μM; P < 0.05) was significantly inhibited by L-NAME (0.5 mM). These results demonstrate that in porcine ciliary processes the increase in nitrite production induced by isoproterenol, forskolin, or 8-bromo-cAMP is mainly caused by the activity of an NOS (Fig. 2).

**CAMP-Induced Nitrite Production and Isoproterenol**

In a concentration-dependent manner, the increase in nitrite production induced by isoproterenol (10 μM) was significantly (P < 0.05) reduced by increasing concentrations of the nonselective β-adrenergic receptor antagonist propranolol (0.1, 1, and 10 μM). In contrast, propranolol had no significant inhibitory effect on the nitrite production evoked by forskolin (1 μM) or 8-bromo-cAMP (10 μM). These results show that in porcine ciliary processes the increase in nitrite production...
induced by isoproterenol can be inhibited by a β-blocker such as propranolol (Fig. 3).

cAMP-Induced Nitrite Production and KT 5720
The increase in nitrite production evoked by isoproterenol (10 μM) was significantly \( (P < 0.05) \) inhibited by different concentrations of the specific cAMP-dependent protein kinase A (PKA) inhibitor KT 5720 (0.1 and 1 μM), but not by the specific cGMP-dependent protein kinase G (PKG) inhibitor KT 5823 (1 μM). In a similar manner, the nitrite production induced by 8-bromo-cAMP (10 μM) was significantly \( (P < 0.05) \) inhibited by the PKA inhibitor KT 5720 (1 μM) but not by the PKG inhibitor KT 5823 (1 μM). Therefore, in porcine ciliary processes, it appears that the increase in nitrite production evoked by isoproterenol and 8-bromo-cAMP is linked to the activity of PKA (Fig. 4).

cAMP-Production in Porcine Ciliary Processes
In isolated porcine ciliary processes, when compared with control experiments (16 ± 1.8 picomoles/mg protein), isoproterenol (10 μM: 30 ± 2.7 picomoles/mg protein) and forskolin (10 μM: 74 ± 8.5 picomoles/mg protein) significantly increased cAMP concentration \( (n = 5; \ P < 0.05 \) and \( P < 0.001 \), respectively). Furthermore, the increase in cAMP production induced by isoproterenol, but not by forskolin, was signifi-

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933583/)  
**Effect of isoproterenol (a β-adrenergic receptor agonist and adenylylcyclase activator), forskolin (an adenylylcyclase activator), and 8-bromo-cAMP (a stable and lipophylic cAMP analog) on the production of nitrite from isolated porcine ciliary processes. Nitrite production was significantly increased by isoproterenol and forskolin, which enhance intracellular production of cAMP, and by 8-bromo-cAMP, which mimics cAMP. Analysis of variance with Bonferroni’s comparison versus 0 μM: **\( P < 0.01 \), ***\( P < 0.001 \).**

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933583/)  
**Effect of the nitric oxide (NO) formation inhibitor L-NAME on the production of nitrite induced by isoproterenol (10 μM), forskolin (1 μM), or 8-bromo-cAMP (10 μM) from isolated porcine ciliary processes. The increase in nitrite production was significantly inhibited by L-NAME, showing the activity of an NOS. D-NAME is the dextrogyre nonmetabolized isoform of L-NAME. Analysis of variance with Bonferroni’s comparison versus 0 μM: **\( P < 0.01 \), ***\( P < 0.001 \).**
cause the increase in nitrite production could be blunted by KT 5720 (a PKA inhibitor) or L-NAME (an inhibitor of NO formation).

Stimulation of β-adrenergic receptors can lead to the activation of a membrane-bound adenylylcyclase and to an increase of intracellular cAMP. Evidence of such a mechanism, which is known to occur in ciliary processes, could also be observed in our porcine ciliary process preparations: the β-adrenergic receptor agonist isoproterenol increased cAMP production, an effect prevented by the nonselective β-adrenergic receptor antagonist propranolol.

**DISCUSSION**

This study shows that drugs that activate adenylylcyclase (isoproterenol, forskolin) or mimic cAMP (8-bromo-cAMP) increase the production of nitrite in isolated porcine ciliary processes. The effect appears to be linked to the activity of a cAMP-dependent protein kinase (PKA) and an NOS, because the increase in nitrite production could be blunted by KT 5720 (a PKA inhibitor) or L-NAME (an inhibitor of NO formation).

Stimulation of β-adrenergic receptors can lead to the activation of a membrane-bound adenylylcyclase and to an increase of intracellular cAMP. Evidence of such a mechanism, which is known to occur in ciliary processes, could also be observed in our porcine ciliary process preparations: the β-adrenergic receptor agonist isoproterenol increased cAMP production, an effect prevented by the nonselective β-adrenergic receptor antagonist propranolol.
Recently, we reported that β-adrenergic receptor activation increases NO production in isolated porcine ciliary processes. The present study further showed that the second-messenger cAMP was involved in this process. Indeed, not only the β-adrenergic receptor agonist isoproterenol (which enhances cAMP production) but also forskolin (an adenyllyclase activator) or 8-bromo-cAMP (a stable and membrane-permeable cAMP analog) increased NO production in this tissue. This effect appears to reflect the activity of PKA, because the increase in NO production evoked by isoproterenol or 8-bromo-cAMP could be blunted by the PKA inhibitor KT 5720, but not by the PKG inhibitor KT 5823. Consistent with these results are several observations made in the literature of the modulation of NOS activity and/or expression by cAMP or PKA.

Isoproterenol, forskolin, and 8-bromo-cAMP increased nitrite production in a dose-dependent manner until a maximum level of production was reached. When higher concentrations of these drugs were used, for a reason we cannot yet explain the increase in nitrite production was reduced.

Until now, several NOS isoforms have been identified that are responsible for the production of NO in many different types of cells (e.g., vascular endothelial cells, neurons, epithelial cells, macrophages). In the present study we did not investigate which NOS isoform or which type of cells (ciliary epithelial cells, smooth muscle cells, endothelial cells, or neuronal cells) were involved in the NO production observed in porcine ciliary processes.

It has been reported that in the kidney, the trachea, or the colon NO is involved in transepithelial ionic and/or fluid transport. The present observation that activation of the β-adrenoceptor–cAMP pathway increases NO production, raises the possibility that NO could, to a certain extent, be involved in the process of aqueous humor formation.

Acknowledgments
The authors thank Andreas Schötzau for his help and advice in the statistical analysis of the data.

References
HLA-B27 Subtypes and HLA Class II Alleles in Japanese Patients with Anterior Uveitis

Yasubiro Konno, Jiro Numaga, Naoyuki Tsuchiya, Atsuko Ogawa, S. M. Monowarul Islam, Manabu Mochizuki, Hiroshi Mitsui, Hiromi Oda, and Hiroo Maeda

PURPOSE. Some patients with anterior uveitis (AU) have ankylosing spondylitis (AS) and are HLA-B27 class I-positive. The purpose of this study was to investigate whether there are differences in HLA at the allele level among each group of patients with AU.

METHODS. Seventy-three patients with AU were studied. They were classified into three groups: 31 with AS-associated AU, 14 with HLA-B27-associated AU, and 28 with idiopathic AU. Three control groups without AU were used: 138 random subjects, 33 HLA-B27-positive healthy subjects, and 19 HLA-B27-positive patients with AS. DRB1 and DQB1 genotyping was performed using polymerase chain reaction (PCR)-single-strand conformation polymorphism (PCR-SSCP) and PCR-restriction fragment length polymorphism. HLA-B27 subtype was determined by PCR-SSCP.

RESULTS. There was no difference in the frequency of any class I antigen except HLA-B27 among the patients studied. The frequencies of HLA-DR12 in AS-associated AU and HLA-DR1 in HLA-B27-associated AU showed an increase. In HLA-B27-associated AU, DRB1*0101 and DQB1*0501 were increased compared with HLA-B27-positive control subjects. When HLA-B27 subtype distribution was compared among the groups, the proportion of B*2704 was significantly lower in HLA-B27-associated AU (P = 0.037), however, such a difference was not present in AS-associated groups.

CONCLUSIONS. These results indicated that B*2704 seemed to be less susceptible to AU compared with B*2705 in Japanese subjects. The increase of HLA-DR12 and HLA-DR1 in AU may be caused by linkage disequilibrium with B*2704 and B*2705, respectively. (Invest Ophthalmol Vis Sci. 1999;40:1838–1844)

From the Departments of Ophthalmology and Orthopedics, Faculty of Medicine, and the Department of Human Genetics, Graduate School of Medicine, University of Tokyo; the Japanese Red Cross Central Blood Center, Tokyo; the Department of Ophthalmology, Faculty of Medicine, Kurume Medical College; the Department of Orthopedics, Mitsui Memorial Hospital; and the Blood Transfusion Service, Saitama Medical Center, Saitama Medical School, Japan. Supported in part by a Grant-in-Aid for Scientific Research (C) No. 07671903 from the Ministry of Education, Science and Culture, Japan. Submitted for publication September 15, 1998; revised February 11, 1999; accepted March 3, 1999.

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Reprint requests: Jiro Numaga, 3-2-1-214, Nishigahara, Kitaku Tokyo 114-0024, Japan.

Anterior uveitis (AU) refers to inflammation mainly involving the anterior segment of the eye, namely the iris and the ciliary body. It develops in some patients as a complication of ankylosing spondylitis (AS), Reiter’s syndrome, juvenile rheumatoid arthritis, or psoriatic arthritis. They are named and classified by the criteria based on systemic diseases—for example, AS-associated AU.

Anterior uveitis is closely associated with HLA-B27. Because this association is observed regardless of the presence of AS, HLA-B27-associated AU appears to be a distinct clinical entity. A substantial proportion of cases of AU are of unknown origin in Japan and are termed idiopathic AU. Although some of the clinical features are different, it is difficult to distinguish these three groups solely from ocular features and symptoms.

The incidence of AU in Japanese people is less frequent than in whites. In the outpatient clinic of Tokyo University Hospital, 2.4% of all outpatients attending an ophthalmology clinic received a diagnosis of uveitis, and approximately 25% of those were diagnosed of AU. In contrast, AU is the most common form of uveitis in whites, accounting for approximately 75% of cases. HLA-B27 shows high frequency, even in Japanese patients with AS and AU, although only 0.8% of the general Japanese population possesses HLA-B27, compared with 4% to 13% of whites. Only limited information has been available concerning HLA-B27 and AU in Japanese people, because of the difficulty in collecting a sufficient number of samples.

In this study, a substantial number of Japanese patients with AS-associated AU, HLA-B27-associated AU, and idiopathic AU were investigated to determine whether there are differences in the frequency of HLA-B27 subtypes or HLA class II alleles among the three groups.

METHODS

Subjects

Seventy-three patients with AU were studied. The diagnosis of AU was determined clinically by ophthalmologists in the uveitis clinic according to the criteria recommended by Rothova et al. Ankylosing spondylitis was diagnosed according to New York criteria by orthopedic medical specialists. The patients were classified into three groups according to the criteria of Nussenblatt et al. Thirty-one patients (27 men and 4 women) were classified as having AS-associated AU, 14 patients (8 men and 6 women) as having HLA-B27-associated AU, and 28 patients (11 men and 17 women) as having idiopathic AU. Patients with Fuchs’ heterochromic iridocyclitis, Posner-Schlossman’s syndrome, ulcerative colitis, and Crohn’s disease were excluded because these conditions were considered to constitute distinct entities. The average age of AU onset was 40.5 ± 11.4 years (in men) and 43.7 ± 15.4 years (in women). All these patients were of Japanese origin and unrelated to one another. They were diagnosed and treated in the University of Tokyo Hospital, Mitsui Memorial Hospital, and Kurume Medical College Hospital during the period from May 1992 through June 1997. Two groups of healthy control subjects were also studied, which included 138 non-HLA matched subjects (random control subjects) and 33 HLA-B27-positive subjects. For the reference of another group of HLA-B27 subtype, 19 patients with AS without AU were included in this study. They were the blood donors and staff at Saitama Medical School, the
Japanese Red Cross Central Blood Center, and the Japanese Red Cross Nagano Blood Center. The study was approved by the Ethics Committee of the University of Tokyo School of Medicine. Informed consent was obtained from each subject before participation in the study. The tenets of the Helsinki Declaration were followed.

HLA Genotyping

Venous blood was obtained from each patient and control subject, and the serologic HLA tissue typing for class I and II specificity was performed by the standard National Institutes of Health complement-dependent microlymphocyte toxicity test. Genomic DNAs were extracted from peripheral blood using the phenol-chloroform method. HLA-B27 alleles were detected by nested polymerase chain reaction (PCR)–single-strand conformation polymorphism (SSCP). The PCR reaction was conducted with DNA polymerase (AmpliTaq; Perkin Elmer Cetus, Norwalk, CT). Briefly, the B locus was amplified with the sense and antisense primer pairs described in Table 1. The reaction mixture was subjected to 30 cycles of 22 seconds at 94°C for denaturation, 50 seconds at 65°C for primer annealing, and 30 seconds at 72°C for extension in an automated thermal cycler (Perkin Elmer Cetus). Then, B*27 exons 2 and 3 were amplified with specific primers for each (Table 1) from the diluted B locus products. The reaction mixture was subjected to 30 cycles of 10 seconds at 96°C, 20 seconds at 64 to 67°C, and 20 seconds at 70°C for extension. Each of the amplified HLA-B27 products was detected by SSCP, as previously described. Briefly, 2.5 μl of PCR products was diluted with 10 μl SSCP buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). These samples were boiled 5 minutes at 95°C for denaturation, cooled in ice water and applied to the 8% to 10% polyacrylamide gel. The samples were subjected to electrophoresis in 0.5× Tris-borate-EDTA buffer (0.5× TBE) at 20 to 30 mA for 2 to 3 hours. The SSCP gel was visualized with silver staining using a commercial silver staining kit (Bio-Rad, Hercules, CA), and the difference of the pattern was compared with reference samples. A few samples in each subtype group were confirmed for the allele by sequencing-based typing.

HLA-DRB1 and HLA-DQB1 genotyping was performed by PCR-SSCP and PCR-restriction fragment length polymorphism (RFLP), as previously described. Briefly, DRB1 and DQB1 PCR was performed with group-specific primer pairs for the selective amplification of each second exon. The sequences of the primers are shown in Table 2. The PCR reaction was conducted with DNA polymerase (AmpliTaq) or thermally activated DNA polymerase (AmpliTaq Gold (both from Perkin Elmer Cetus). The PCR condition for DRB1 was 30 cycles of 1 minute at 96°C, 1 minute at 54°C to 61°C, and 30 to 60 seconds at 72°C in an automated thermal cycler. The PCR condition for DQB1 was 30 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 30 seconds at 72°C in an automated thermal cycler. SSCP was performed as described in HLA-B27 genotyping. RFLP was performed for the samples that were difficult to genotype with SSCP only. Five microliters of the PCR product was incubated and digested with the recommended buffer and appropriate restriction enzyme at 37°C for 2 to 3 hours. Samples were subjected to electrophoresis in 8% to 12% polyacrylamide gel in 0.5× TBE buffer at 20 to 30 mA for 30

| Table 1. Primer Sets to Amplify B Locus, HLA-B27 Exon 2 and Exon 3 |
|------------------|------------------|------------------|
| **Locus/Exon**   | **Sequence (5’–3’)** |
| **Forward**      | **Reverse**      |
| B locus          | 5Bln1–57         | 3Bln3–37         |
| HLA-B27 exon2    | E40s             | E90as            |
| exons            | 3Bln3–37         | 3Bln3–37         |
| (B*2707, B*2711) | E91As            | E136as           |
|                  | E91Bs            | E181as           |

| Table 2. Group-Specific Primer Sets to Amplify DRB1 and DQB1 Allele |
|------------------|------------------|------------------|
| **DRB1**         | **Sequence (5’–3’)** |
| for DR1          | GGTTGCTGGAAGAGATGCATCT |
| for DR2          | TTCTGTGGCAGCTAAGAGG |
| for DR4          | GTTTCTGGACAGCTTAAC |
| for DR7          | AGTTCTGGAAAGACCTTCTT |
| for DR9          | GAAGCAGGATAAGTITGTG |
| for DR10         | GGTTGCTGGAAGAAGCAGCC |
| for DR3, 5, 6, 8 | AGCTTTCTTGAGAGTACCTACG |
|                  | CCGCTGCACTGTGAAGCTC |
| **DQB1**         | **Sequence (5’–3’)** |
| for DQ1          | GH28NL           | GB202            |
| for DQ2, 3, 4    | GH28NL           | GB204            |

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minutes. Digested fragments were visualized by staining with ethidium bromide.

Statistical Analysis

The $\chi^2$ method with the continuity correction and Fisher’s exact probability test were used for data analysis, when appropriate. Statistical significance was defined as $P < 0.05$, and $P$ was corrected ($Pc$) for the member of comparison where necessary. Odds ratios were calculated by Haldane’s modification of Woolf’s method. Briefly, $(a \times d)/(b \times c)$, where $a$, $b$, $c$, and $d$ are the numbers of marker-positive patients, marker-negative patients, marker-positive control subjects, and marker-negative control subjects, respectively.

RESULTS

Table 3 shows HLA class I phenotype frequencies in the AS-associated AU group, the HLA-B27-associated AU group, the idiopathic AU group, and random control subjects. The most striking difference was seen in HLA-B27: 93.5% (29/31) in the
As-associated AU group, 100% (14/14) in the HLA-B27–associated AU group, 0% (0/28) in the idiopathic AU group, and 0.7% (1/138) in random control subjects. No other class I antigens showed significant differences when P was corrected for the number of comparisons. Table 4 shows the HLA class II phenotype frequencies among the same groups. HLA-DR1 showed significantly increased frequency (50.0%) in the HLA-B27–associated AU group compared with the control subjects (8.0%). The frequency of HLA-DR12 was significantly increased in the AS-associated AU group (22.6%) compared with the control group (4.3%). No differences were found between idiopathic AU and the random control groups (Tables 3, 4).

We next focused on the HLA-B27–positive subjects to avoid the bias caused by the possession of HLA-B27, and compared HLA class II alleles and HLA-B27 subtypes at the sequence level among AS-associated AU, HLA-B27–associated AU, and HLA-B27–positive healthy subjects (Tables 5, 6). Total number of the HLA-B27–positive subjects was 29 in the AS-associated AU group, 14 in the HLA-B27–associated AU group, and 33 in healthy control subjects. No differences were found in the distribution of HLA class I among these subjects (data not shown). HLA-DRB1*0101 and DQB1*0501 were shown to be slightly increased in the HLA-B27–associated AU group, although the difference was no longer significant when the p value was corrected for the number of alleles. The positivity of other DRB1 and DQB1 alleles did not show significant differences from HLA-B27–positive control subjects.

Table 7 shows the distribution of HLA-B27 subtypes among the three groups and in patients with AS without AU. Of interest, the proportion of B*2704 positive subjects was significantly smaller in the HLA-B27–associated AU group (50.0%) than in control subjects (81.8%; P = 0.037). Such a difference was not observed in patients with AS, regardless of the presence of AU.

**DISCUSSION**

Although the role of HLA-B27 in the susceptibility to AS has been investigated in various ethnic groups, little is known about the ethnic difference in the relationship between HLA-B27 and AU. One of the reasons may be the difficulty in making the diagnosis of AU after the inflammation has subsided, because the severe phase of AU usually disappears in several weeks. HLA-B27 is considered to be associated with both AS and AU, but HLA-B27 seems to influence the susceptibility to AS more strongly than to AU.4,9 It is possible that the analysis of genetic factors in patients with AS-associated AU are strongly influenced by the presence of AS. Therefore, genetic factor(s) primarily involved in AU should be analyzed in patients who have AU without AS to avoid the influence of genetic factor(s) in AS.

The major finding in this study was the difference in the susceptibility to AU among HLA-B27 subtypes in Japanese people. Among the 12 HLA-B27 subtypes (B*2701–B*2712 thus far identified), B*2711 has been reported only in Japanese people but not in various ethnic populations.10 Other B*27 subtypes, B*2701, B*2702, B*2703, B*2706, B*2707, B*2708, B*2709, B*2710, and B*2712, were not detected in our Japanese sub-

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**Table 4. HLA Class II Antigen Frequencies in AS-Associated, B27-Associated, Idiopathic AU, and Random Control Groups**

<table>
<thead>
<tr>
<th>Class II Antigen</th>
<th>AS-Associated n = 31 (%)</th>
<th>B27-Associated n = 14 (%)</th>
<th>Idiopathic n = 28 (%)</th>
<th>Random Controls n = 138 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR1</td>
<td>8 (25.8)</td>
<td>7 (50.0)*</td>
<td>5 (17.9)</td>
<td>11 (8.0)</td>
</tr>
<tr>
<td>DR2</td>
<td>10 (32.3)</td>
<td>1 (7.1)</td>
<td>10 (35.7)</td>
<td>47 (34.1)</td>
</tr>
<tr>
<td>DR3</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>DR4</td>
<td>8 (25.8)</td>
<td>5 (35.7)</td>
<td>9 (32.1)</td>
<td>60 (43.5)</td>
</tr>
<tr>
<td>DR6</td>
<td>7 (22.6)</td>
<td>2 (14.3)</td>
<td>9 (32.1)</td>
<td>47 (34.1)</td>
</tr>
<tr>
<td>DR7</td>
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<td>0 (0)</td>
<td>1 (3.6)</td>
<td>2 (1.4)</td>
</tr>
<tr>
<td>DR8</td>
<td>12 (38.7)</td>
<td>5 (35.7)</td>
<td>9 (32.1)</td>
<td>54 (42.6)</td>
</tr>
<tr>
<td>DR9</td>
<td>4 (12.9)</td>
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<td>5 (17.9)</td>
<td>41 (30.7)</td>
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<td>0 (0)</td>
<td>0 (0)</td>
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<tr>
<td>DR12</td>
<td>7 (22.6)†</td>
<td>2 (14.3)</td>
<td>4 (14.3)</td>
<td>6 (4.3)</td>
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<tr>
<td>DR13</td>
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<td>2 (7.1)</td>
<td>22 (15.9)</td>
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<td>4 (14.3)</td>
<td>15 (10.9)</td>
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<td>DRHB5</td>
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<td>1 (7.1)</td>
<td>5 (17.9)</td>
<td>14 (10.1)</td>
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</table>

* P = 0.00021, Pc = 0.0035; odds ratio = 11.5 compared with random controls.
† P = 0.00283, Pc = 0.048; odds ratio = 6.4 compared with random controls.
A difference in the susceptibility to AU among HLA-B27 subtypes was not reported in whites. Because B*2705 and B*2702 account for the majority of HLA-B27 subtypes in whites, it can be interpreted that there is no difference in the susceptibility to AU between B*2702 and B*2705. In this study, B*2704 was detected significantly less frequently than B*2705.

### Table 5. DRB1 Allelic Frequencies among the HLA-B27–Positive Groups with AS-Associated AU and HLA-B27–Associated AU and Controls

<table>
<thead>
<tr>
<th>DRB1*</th>
<th>AS-Associated AU</th>
<th>B27-Associated AU</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 29 (</td>
<td>n = 14 (%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
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<td>3 (10.3)</td>
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<td>1406</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (9.1)</td>
</tr>
<tr>
<td>1403</td>
<td>0 (0)</td>
<td>1 (7.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1412</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0701</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0803</td>
<td>9 (31.0)</td>
<td>7 (50.0)*</td>
<td>10 (30.3)</td>
</tr>
<tr>
<td>0802</td>
<td>1 (3.4)</td>
<td>2 (14.3)</td>
<td>1 (3.0)</td>
</tr>
<tr>
<td>0801</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0901</td>
<td>4 (13.8)</td>
<td>3 (21.4)</td>
<td>7 (21.2)</td>
</tr>
<tr>
<td>1001</td>
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</tbody>
</table>

*P = 0.0091, Pc = 0.18; odds ratio = 7.3 compared with controls.

### Table 6. DQB1 Allelic Frequencies among the HLA-B27–Positive Groups with AS-Associated AU and HLA-B27–Associated AU and Controls

<table>
<thead>
<tr>
<th>DQB1*</th>
<th>AS-Associated AU</th>
<th>B27-Associated AU</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 29 (</td>
<td>n = 14 (%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>0501</td>
<td>9 (31.0)</td>
<td>7 (50.0)†</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>0502</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>0503</td>
<td>2 (6.9)</td>
<td>0 (0)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>0601</td>
<td>15 (51.7)</td>
<td>3 (21.4)</td>
<td>7 (43.8)</td>
</tr>
<tr>
<td>0602</td>
<td>2 (6.9)</td>
<td>0 (0)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>0603</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0604</td>
<td>4 (13.8)</td>
<td>1 (7.1)</td>
<td>0 (0)</td>
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<td>0605/9</td>
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<td>0 (0)</td>
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<td>0201</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0301</td>
<td>10 (34.5)</td>
<td>4 (28.6)</td>
<td>5 (31.3)</td>
</tr>
<tr>
<td>0302</td>
<td>5 (17.2)</td>
<td>1 (7.1)</td>
<td>2 (12.5)</td>
</tr>
<tr>
<td>0303</td>
<td>3 (10.3)</td>
<td>5 (21.4)</td>
<td>4 (25.0)</td>
</tr>
<tr>
<td>0401</td>
<td>4 (13.8)</td>
<td>5 (35.7)</td>
<td>7 (43.8)</td>
</tr>
<tr>
<td>0402</td>
<td>0 (0)</td>
<td>1 (7.1)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

†P = 0.012, Pc = 0.17; odds ratio = 15.0 compared with controls.
in the HLA-B27–associated AU group than in HLA-B27–positive control subjects. A similar finding was recently reported by Goto et al. Because both of the subjects were of Japanese ancestry, and none of the patients with AU in their study had AS, their data were combined with ours and were reanalyzed (Table 8). The result also shows the difference in HLA-B27 subtype distribution between HLA-B27–associated AU and HLA-B27–positive control subjects.

In the case of AS, B*2706 and B*2709 were reported to have negative associations. It is speculated that differences in the affinity between potential pathogenic peptides and the HLA-B27 peptide-binding pocket may influence the degree of susceptibility conferred by each subtype. This affinity may be influenced by the characteristics of HLA-B27 amino acid residues outside the B pocket, especially in the F and D/E pockets. B*2704, B*2706, and B*2710 possess the same residue at 152 in pocket E, which is different from other HLA-B27 subtypes. B*2710, which is different at only 152 amino acid residues from B*2705, were reported to have weak affinity with T-cell receptor. From these data, it could be speculated that B*2704 has a relatively high affinity with the AU-specific peptides or weak antigen recognition for T-cell receptor. Because the eye is known to be an immunologically privileged site, it is likely that antigenic peptides presented by HLA-class I are different from those in other tissues, such as spine.

We also detected a slight differences in frequencies of HLA-DR12 and HLA-DR1. HLA-DR12 was increased in AS-associated AU compared with incidence in random control subjects but failed to show increased frequency when compared with HLA-B27–positive control subjects, suggesting that the increase of HLA-DR12 was caused by linkage disequilibrium with HLA-B27. Conversely, slight increases in the proportions of DRB1*0101 and DQB1*0501 were present in HLA-B27–associated AU, even compared with that in HLA-B27–positive control subjects. It is known that DRB1*0101 and DQB1*0501 are in tight linkage, and the linkage disequilibrium between B*2705-DR1 has been observed in populations world-wide.

In fact, we recently reported that HLA-DRB1*1202 and HLA-DQB1*0101 are in linkage disequilibrium between HLA-B*2704 and HLA-B*2705, respectively, in the Japanese population. Based on these findings, it is thought that the difference in DR12 and DR1 frequency was caused by the linkage disequilibrium with HLA-B27 subtypes. However, it remains to be determined which of the HLA-B*2705 and DRB1*0101-DQB1*0501 has the primary effect on the susceptibility to AU.

No HLA differences were found in those with idiopathic AU compared with random control subjects. It remains unclear whether the pathogenesis of idiopathic AU is similar to HLA-B27–associated AU, despite the similarity in clinical features. The relationship between HLA-B27 subtypes and development of AS or AU suggests the possibility of graded susceptibility to both diseases, instead of the all-or-nothing rule. This scenario could be accepted easily under conditions in which several kinds of pathogenic peptides with different affinity for the HLA-B27 subtypes are involved in the pathogenesis. The disease susceptibility differences could be explained not only with HLA-B27 subtypes, but the binding affinities of pathogenic peptides may also be taken into consideration.

### Acknowledgments

The authors thank those who participated in this study; Satoshi Saito, Nagano Red Cross Blood Center, who kindly shared HLA-B27 healthy control DNA samples; and Ranko Hirata, Blood Transfusion Service, Saitama Medical Center, who performed HLA serologic typing.

### References

5. Imanishi T, Akaza T, Kimura A, Tokunaga K, Gojobori T. Allelic and haplotype frequencies for HLA and compliment loci in various

### Table 7. HLA-B27 Subtype Distribution in HLA-B27–Positive Subjects

<table>
<thead>
<tr>
<th>HLA-B27 Subtype</th>
<th>AS-Associated AU</th>
<th>B27-Associated AU</th>
<th>AS without AU</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 29 (%)</td>
<td>n = 14 (%)</td>
<td>n = 19 (%)</td>
<td>n = 33 (%)</td>
</tr>
<tr>
<td>B*2704</td>
<td>21 (72.4)</td>
<td>7 (50.0)*</td>
<td>17 (89.5)</td>
<td>27 (81.8)</td>
</tr>
<tr>
<td>Other B*27 subtypes†</td>
<td>8 (27.6)</td>
<td>7 (50.0)</td>
<td>2 (10.5)</td>
<td>6 (18.2)</td>
</tr>
</tbody>
</table>

* P = 0.037; odds ratio = 4.5 compared with controls.
† All subjects in this group possessed B*2705 except one control who possessed B*2711.

### Table 8. HLA-B27 Subtype Distribution in HLA-B27–Positive Subjects: Combined Data from Present Study and Results from Goto et al.

<table>
<thead>
<tr>
<th>HLA-B27 Subtype</th>
<th>B27-Associated AU</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 31 (%)</td>
<td>n = 53 (%)</td>
</tr>
<tr>
<td>B*2704</td>
<td>17 (54.8)</td>
<td>43 (81.1)</td>
</tr>
<tr>
<td>Other B*27 subtypes</td>
<td>14 (45.2)</td>
<td>10 (18.9)</td>
</tr>
</tbody>
</table>

* P = 0.0101; χ² = 6.6.
Characterization of a Mouse Cx50 Mutation Associated with the No2 Mouse Cataract

Xiaorong Xu and Lisa Ebihara

PURPOSE. Recently, a missense mutation in the mouse connexin 50 (Cx50) gene has been associated with the nuclear opacity 2 (No2) mouse cataract. This missense mutation (D47A) resulted in an aspartate-to-alanine substitution at amino acid position 47 in the first extracellular domain of Cx50. To better understand the role of Cx50 in the pathogenesis of congenital cataract, the functional consequences of the D47A mutation in the Xenopus oocyte expression system were studied.

METHODS. D47A was constructed using polymerase chain reaction (PCR) mutagenesis. Xenopus oocytes were injected with in vitro transcribed cRNA encoding wild-type mouse Cx50 (Cx50wt), wild-type rat Cx46 (Cx46wt), D47A, or combinations of wild-type and mutant connexins. The oocytes were then devitellinized and paired. Gap junctional conductance (Gj) was measured using a dual two-microelectrode voltage-clamp technique.

RESULTS. Homotypic oocyte pairs expressing wild-type Cx50 or Cx46 were well coupled. In contrast, oocytes injected with D47A cRNA did not form gap junctional channels when paired homotypically. To test whether the D47A mutation could interact with wild-type connexins in a dominant negative manner, oocytes were injected with equal amounts of mutant and wild-type connexin cRNA, mimicking the heterozygous condition. Expression of D47A did not inhibit the development of junctional conductance in paired oocytes induced by wild-type Cx50 or Cx46.

CONCLUSIONS. These results indicate that the D47A mutation acts as a loss-of-function mutation without strong dominant inhibition. In No2 mice, the mutation would be predicted to result in a reduction in intercellular communication, leading to cataractogenesis. It may also cause other qualitative changes such as a change in permeability for small molecules.

Gap junctional channels are intercellular pathways between adjacent cells for the exchange of ions and metabolites smaller than 1 kDa. The gap junction channel is made of two connexins, each contributed separately by two adjoining cells. The hemichannels are composed of six subunits called connexons. The connexins belong to a multigene family composed of at least 14 members. Three connexins have been identified in the rodent lens: Cx43, Cx46, and Cx50. Mouse connexin 50 (mCx50) is expressed only in the lens, where it forms gap junctional channels between fiber cells. Connexin 46 is also found in lens fiber–fiber gap junctions, whereas connexin 43 is expressed in lens epithelial cells. Mutations in connexins have been linked to several genetic diseases including X-linked Charcot–Marie–Tooth disease (CMTX), a demyelinating peripheral neuropathy that is associated with mutations in Cx32; hereditary nonsyndromic...
deafness, which is associated with mutations in Cx26; and visceral atrial heterotaxia syndromes, which are associated with mutations in Cx43. Recently, a missense mutation in the mouse connexin 50 gene (Gja50) has been associated with the nuclear opacity 2 (No2) mouse cataract, a congenital hereditary bilateral cataract that is inherited in a semidominant manner. This missense mutation results in a substitution of aspartic acid-to-alanine at amino acid position 47 in the first putative extracellular domain of Cx50. To understand better the role of Cx50 in the pathogenesis of congenital cataract, we studied the functional consequences of the Cx50D47A mutation by testing its ability to induce gap junctional coupling between paired oocytes.

**Materials and Methods**

**Mutagenesis**

cDNAs encoding mouse Cx50 and rat Cx46 in the pSP64TII vector were provided by Thomas W. White and Daniel A. Goodenough (Harvard University, Boston, MA). To generate Cx50D47A, two primers corresponding to adjacent regions in the E1 domain of Cx50 were synthesized. The sense primer, 5’-CTGAGCACAATCGATTTGATGCAACACC-3’, corresponding to nucleotides from 406 to 434, contained nucleotides encoding amino acid 47 to 56 of Cx50, with nucleotide 406 changed from A to C, resulting in the conversion of aspartic acid 47 to alanine. The antisense primer, 5’-CGGCCCAACAAAATCCGCCCT-3’, corresponding to nucleotides from 405 to 386, corresponds to amino acids 40 to 47 of Cx50. Cx50 in the SP64T vector was amplified using a commercial kit (LA PCR; Takara Shuzo, Ostsu, Japan) according to the manufacturer’s protocol. The polymerase chain reaction (PCR) conditions were as follows: 1 minute at 94°C; 25 cycles at 98°C for 20 seconds and 68°C for 10 minutes; and 1 cycle at 72°C for 10 minutes. The PCR-amplified product was digested with the restriction enzyme, DpnI, to select against the parental, nonreplicated DNA. Subsequently, the PCR-amplified product was purified with a PCR purification kit (QIAquick; Qiagen, Chatsworth, CA), resolved on a sodium dodecyl sulfate–containing 9% polyacrylamide gel and transferred to nitrocellulose. The western blots were probed with the anti-Cx50 monoclonal antibody 6-4-2-C6 (kindly provided by Viviana Berthoud and Eric Beyer, University of Chicago, IL). The recombinant plasmid DNA was linearized with the restriction enzyme, SalI. cRNAs were in vitro transcribed with SP6 polymerase (mMessage mMACHINE kit; Ambion, Austin, TX) following the manufacturer’s protocol. The transcripts were purified on a 0.5-1 Sephadex column (Boehringer Mannheim, Indianapolis, IN) to remove unincorporated rNTPs, precipitated with isopropanol, and resuspended in diethyl pyrocarbonate-treated water. The cRNA was quantitated by measuring the absorbance at 260 nm and stored as 3-μl aliquots at −80°C.

**Preparation of Xenopus Oocytes**

Female *Xenopus laevis* was anesthetized, and a partial ovariectomy was performed. The frogs were maintained and treated in accordance with National Institutes of Health guidelines and with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The oocytes were treated with 10 mg/ml collagenase type IA (Sigma, St. Louis, MO) for 20 minutes, manually defolliculated, and injected with an oligonucleotide antisense to endogenous Cx38, as previously described. The oocytes were then injected with 3 to 4 ng cRNA for mouse Cx50, mouse Cx50D47A, or rat Cx46, either alone or in combination, and allowed to incubate for an additional 6 to 48 hours. Then the oocytes were devitellinized and paired as previously described. Electrophysiological measurements were performed 6 to 18 hours after pairing.

**Western Blot Analysis of Connexin Proteins**

Plasma membrane-enriched preparations of *Xenopus* oocytes were prepared as previously described. The proteins were resolved on a sodium dodecyl sulfate–containing 9% polyacrylamide gel and transferred to nitrocellulose. The western blots were probed with the anti-Cx50 monoclonal antibody 6-4-B2-C6 (kindly provided by Viviana Berthoud and Eric Beyer, University of Chicago, IL). The primary antibody was detected with alkaline phosphatase-conjugated goat anti-mouse Ig (Boehringer-Mannheim, Indianapolis, IN).

**Table 1. Conductance of Xenopus Oocyte Pairs Injected with Mouse Cx50 Wild-type and/or Cx50D47A Mutant cRNA**

<table>
<thead>
<tr>
<th>Cell Injected</th>
<th>Mean Conductance ± SEM*</th>
<th>Number of Pairs</th>
<th>Expression Time†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.033 ± 0.012</td>
<td>20</td>
<td>2 days</td>
</tr>
<tr>
<td>Control</td>
<td>0.052 ± 0.008</td>
<td>12</td>
<td>o/n</td>
</tr>
<tr>
<td>Cx50wt</td>
<td>29.1 ± 5.77</td>
<td>14</td>
<td>2 days</td>
</tr>
<tr>
<td>Cx50wt</td>
<td>9.43 ± 5.10</td>
<td>4</td>
<td>o/n</td>
</tr>
<tr>
<td>D47A</td>
<td>0.017 ± 0.003</td>
<td>5</td>
<td>2 days</td>
</tr>
<tr>
<td>D47A</td>
<td>0.047 ± 0.016</td>
<td>11</td>
<td>o/n</td>
</tr>
<tr>
<td>Cx50wt+D47A</td>
<td>25.2 ± 12.8</td>
<td>5</td>
<td>2 days</td>
</tr>
<tr>
<td>Cx50wt+D47A</td>
<td>6.12 ± 2.08</td>
<td>3</td>
<td>o/n</td>
</tr>
</tbody>
</table>

The total amount of cRNA injected into each oocyte was held constant. Therefore, oocytes co-injected with mutant and wild-type Cx50 cRNA at a 1:1 ratio would be expected to have junctional conductances that were reduced by 50% compared with conductance in oocytes injected with wild-type Cx50 alone, if the D47A mutation does not have a dominant negative effect.

* Data are expressed in microsiemens.

† Oocytes were injected with wild-type or mutant Cx50 cRNA and allowed to incubate at 18°C, either overnight or for 2 days before pairing.
Electrophysiological Measurements and Analysis

Dual two-microelectrode voltage-clamp recordings of gap junctional channels were performed (Axoclamp 2A and a Geneclamp 500 amplifier; Axon Instruments, Foster City, CA). The current and voltage electrodes were filled with 3 M KCl and had resistances of 0.1 to 0.5 MΩ. The tips of the electrodes were back filled with 1% agar in 3 M KCl to prevent KCl from leaking out of the electrodes and damaging the oocytes. Data acquisition and analysis were as performed (Pentium computer equipped with a TL-1 labmaster board and Pclamp6 software; Axon Instruments, Austin, Texas). Currents were filtered at 50 Hz using a four-pole Bessel filter. All experiments were performed at room temperature (22°C–24°C). For simple measurement of gap junctional conductance, both cells of the pair were initially voltage clamped to –40 mV and a 5- to 10-mV pulse was applied to one cell. Under these conditions, the change in current recorded in the second cell would be equal in magnitude and opposite in polarity to the current flowing through the gap junction and could be divided by change in transjunctional voltage to determine junctional conductance, \( G_j \). To evaluate the transjunctional voltage dependence of the gap junctions, transjunctional voltage-clamp steps were applied between ±70 mV in 10-mV increments from a holding potential of –40 mV. The initial and steady state junctional currents were measured at 40 msec and 24 seconds, respectively, after application of the voltage-clamp step. The normalized steady state junctional conductance \( G_j^\prime \) versus transjunctional voltage \( V_j \) relation was determined by normalizing the steady state conductance values to the values at \( ±10 \) mV. The \( G_j^\prime-V_j \) relation was fit to a Boltzmann equation:

\[
G_j^\prime = G_{j\min} + \frac{G_{j\max} - G_{j\min}}{1 + \exp[A*(V_j - V_0)]},
\]

where \( G_j^\prime \) is the steady state conductance, \( G_{j\min} \) is the minimum conductance, \( G_{j\max} \) is the maximum conductance, \( A \) is the cooperativity constant, and \( V_0 \) is the voltage at which the decrease in \( G_j^\prime \) is half maximal. Oocyte pairs with resting membrane potentials more negative than –15 mV were selected for analysis.

### Table 2. Boltzmann Parameters of Normalized Mean Steady State Conductance of Gap Junctional Channels Expressed in Xenopus Oocyte Pairs

<table>
<thead>
<tr>
<th>Cell Injected</th>
<th>( G_{j\max} )</th>
<th>( G_{j\min} )</th>
<th>( A )</th>
<th>( V_0^* )</th>
<th>Number of Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx50wt</td>
<td>1.04</td>
<td>0.16</td>
<td>0.25</td>
<td>22.76</td>
<td>4</td>
</tr>
<tr>
<td>Cx50wt+D47A</td>
<td>1.10</td>
<td>0.18</td>
<td>0.17</td>
<td>20.23</td>
<td>6</td>
</tr>
</tbody>
</table>

Data were fit to a Boltzmann equation: \( G_j = G_{j\min} + (G_{j\max} - G_{j\min})/[1 + \exp(A*(V_j - V_0))] \).

* Data are in millivolts.
RESULTS

Expression of Cx50D47A in Oocyte Pairs

We compared the functional properties of the D47A mutation with those of the wild-type protein by testing their ability to form gap junctional channels in the paired oocyte system. The results of these experiments are summarized in Table 1. Oocytes injected with cRNA for D47A did not induce the formation of gap junctional channels when paired homotypically. In contrast, homotypic oocyte pairs expressing wild-type Cx50 were well coupled. Figure 1A shows typical junctional currents from a homotypic pair expressing wild-type Cx50. The Cx50 gap junctional current rapidly inactivated to a new steady state level on application of transjunctional voltage-clamp steps to potentials greater than ±10 mV. The time course of inactivation became progressively faster at larger transjunctional potentials. Figure 1B shows a plot of the normalized steady state junctional conductance (Gj) versus transjunctional voltage (Vj) relation. Gj declined symmetrically at positive and negative Vj values. The mean Gj–Vj curve (n = 4) could be described by a Boltzmann function with Gjmax = 1.04; Gjmin = 0.16, A = 0.25 and Vj0 = 22.76 mV (Table 2). These values are similar to those previously reported by White et al.17

To test whether the D47A mutation could interact with wild-type Cx50 in a dominant negative manner, we coinjected oocytes with equal amounts of mutant and wild-type Cx50 cRNA, mimicking the heterozygous situation. Expression of D47A did not inhibit the development of junctional conductance in paired oocytes induced by wild-type Cx50 (Table 1). Moreover, the time course of inactivation and the Gj–Vj curve were not altered by coexpression of D47A with wild-type Cx50 (Table 2). These results indicate that the D47A mutation acted as a loss-of-function mutation without having a dominant negative effect.

Effect of Coexpression of Cx50D47A with Wild-type Cx46

Previous biochemical studies have shown that Cx50 forms heteromeric gap junctional channels with Cx46 in lens fiber cells.19,20 Thus, we were interested in determining whether coexpression of the D47A mutant with wild-type rat Cx46 would inhibit gap junctional coupling. Oocyte pairs injected with wild-type rat Cx46, either alone or in combination with wild-type mouse Cx50 cRNA, efficiently made gap junctional channels (Table 3). Oocytes coinjected with Cx46 and D47A mutant cRNA were also well coupled, showing that the D47A mutant did not significantly inhibit the ability of wild-type Cx46 to form gap junctional channels. Figure 3 shows representative junctional current traces and plots of normalized Gj–Vj for homotypic Cx46 and heteromeric (Cx46+Cx50) and (Cx46+Cx50D47A) pairs. The junctional currents recorded for Cx46 pairs displayed voltage sensitivity at larger transjunctional voltages. The Gj–Vj relation decreased symmetrically for Vj of opposite polarities with a Vj0 of 52.86 mV (Table 4). Similar findings for Cx46 gap junctional channels

| Table 3. Conductance of Xenopus Oocyte Pairs Coinjected with Rat Cx46 Wild-type and Mouse Cx50 Wild-type or D47A cRNA |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Cell Injected** | **Mean Conductance ± SEM** | **Number of Pairs** | **Expression Time** |
|                  | Cell 1          | Cell 2          |                  |                  |
| Control          | Control         | 0.046 ± 0.016   | 5                | 2 days           |
| Control          | Control         | 0.026 ± 0.010   | 10               | o/n              |
| Cx46wt+Cx50wt    | Cx46wt+Cx50wt   | 16.8 ± 4.73     | 4                | 2 days           |
| Cx46wt+Cx50wt    | Cx46wt+Cx50wt   | 14.5 ± 7.16     | 9                | o/n              |
| Cx46wt+D47A      | Cx46wt+D47A     | 3.70 ± 3.64     | 3                | 2 days           |
| Cx46wt+D47A      | Cx46wt+D47A     | 4.06 ± 1.37     | 6                | o/n              |
| Cx46wt           | Cx46wt          | 9.03 ± 3.22     | 5                | o/n              |

* Data are in microsiemens.
have been reported by White et al.\textsuperscript{17} The junctional currents recorded from oocyte pairs expressing (Cx46\textsuperscript{1}Cx50D47A) also decayed in a time- and voltage-dependent manner at $V_j$ of $\pm 30$ mV or more. The mean $G_{j0}-V_j$ relation could be described by a Boltzmann function with a $V_0$ of 38.09 mV. In contrast to both Cx46 and (Cx46\textsuperscript{1}Cx50D47A) pairs, (Cx46+Cx50) pairs displayed a much greater sensitivity to voltage. The Boltzmann parameters for the mean $G_{j0}-V_j$ relation were similar to the values for homotypic pairs expressing wild-type Cx50 with a $V_0$ of 25.91 mV.

**DISCUSSION**

This study shows that mouse Cx50D47A acts as a loss-of-function mutation without dominant inhibition. The observa-
tion that the D47A mutation does not have a strong dominant negative effect on wild-type Cx50 or Cx46 suggests that it is unable to coassemble with the wild-type connexins. Alternatively, it is possible that the D47A mutation can coassemble with wild-type connexins and that the function of these heteromeric channels depends on the number and placement of the mutant subunits. Further studies are needed to distinguish between these two possibilities.

The finding that the D47A mutation leads to loss of function is not surprising. The E1 loop of the connexin protein is a highly conserved and functionally important domain in gap junctional coupling and gating. The invariance of the aspartic acid at amino acid position 47 is suggestive of its importance. Mutations at this position would potentially alter the structure of the E1 loop and perturb its ability to dock with an opposing connexon.

The No2 mouse mutation has been described as semidominant because heterozygous mice have a milder form of cataract than do homozygous mice. These observations are consistent with the notion that D47A acts as a loss-of-function mutation without dominant inhibition. Consequently, cataract formation occurs when the amount of wild-type Cx50 is reduced below a critical level, and the severity of the cataract depends on the amount of reduction. In addition, the No2 mice exhibit a reduction in total ocular mass of approximately 30% compared with wild-type, suggesting that the Cx50 gap junctional channels are also involved in the regulation of growth.11 A similar reduction in ocular size and diffuse nuclear opacities has been observed in homozygous Cx50 knockout mice.21 However, no phenotype was observed in heterozygotes suggesting that the effect of the D47A mutation cannot be completely reproduced by knocking out one allele.

Recently, a human congenital zonular pulverulent cataract has been linked to a missense mutation in human Cx50 concomitant with dominant inhibition.23 It would be interesting to determine whether expression of the P88S mutation in mice by homologous recombination would result in a more severe form of cataract than does the D47A mutation.

**Acknowledgments**

The authors thank Jay Pal for reviewing the manuscript and Xiaoqin Liu for technical assistance.

**References**


**Table 4.** Boltzmann Parameters of Normalized Mean Steady State Conductance of Gap Junction Channels Expressed in Xenopus Oocyte Pairs

<table>
<thead>
<tr>
<th>Cell Injected</th>
<th>Cell Injected</th>
<th>G\textsubscript{max}</th>
<th>G\textsubscript{min}</th>
<th>A</th>
<th>V\textsubscript{0}*</th>
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<tr>
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<td>Cx46wt</td>
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<td>0.25</td>
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<tr>
<td>Cx46wt+D47A</td>
<td>Cx46wt+D47A</td>
<td>1.08</td>
<td>0.32</td>
<td>0.09</td>
<td>38.09</td>
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</tbody>
</table>

Data were fit to a Boltzmann equation \( G = G\text{min} + (G\text{max} - G\text{min})/(1 + \exp(A(V - V\text{0}))) \).

* Data are expressed in millivolts.
Human Ocular Vasodynamic Changes in Light and Darkness

Ulf Havelius,1 Flemming Hansen,2 Bengt Hindfelt,3 and Torsten Krakau1

PURPOSE. To determine whether changes in the retinal blood flow in light and darkness occur in humans.

METHODS. The systolic and diastolic flow velocities were measured by color Doppler in the ophthalmic and the central retinal arteries in 12 healthy individuals in light and darkness.

RESULTS. In the ophthalmic artery there was a trend toward lower systolic velocity in darkness compared with that in the light, but there was no change in diastolic velocity. In the central retinal artery the systolic and the diastolic flow velocities were markedly increased in darkness. After re-exposure to light the systolic flow velocity decreased.

CONCLUSIONS. Darkness is associated with increased blood flow velocity in the central retinal artery, probably reflecting increased retinal metabolic demands by the photoreceptors. (Invest Ophthalmol Vis Sci. 1999;40:1850–1855)

It is well known that even slight hypoxemia reduces dark adaptation, probably by interfering with the high energy demands of the photoreceptor complex. Likewise, it has been shown in animal experiments that the retinal consumption of oxygen and glucose are increased in darkness compared with that in the light.1–3 Whether this increase in retinal metabolism is associated with significant changes in retinal blood flow is not clear.4 In the few human studies of retinal blood flow in light and darkness that have been performed, the results have been inconsistent.5–7 To see if changes in the retinal blood flow in light and darkness occur in humans, the arterial flow velocities within the central retinal and ophthalmic arteries were measured by color Doppler technique in healthy subjects in standardized light and in darkness.

METHODS

Subjects

Twelve healthy individuals were examined by color Doppler imaging (5 men and 7 women; 24–51 years of age; mean, 33 years). All participating individuals were anamnestically free from cardiovascular and neurologic diseases, took no medications, and were nonsmokers. They were ophthalmologically healthy (i.e., had normal vision, intraocular pressure [IOP], dark adaptation, and pupillary reactions).

Test Procedure

All subjects were identically examined by color Doppler imaging (CDI) in supine position, shielded from the monitor. The measurements were performed by an experienced laboratory technician. Blood flow velocities in the ophthalmic and central retinal arteries were measured in each pair of eyes seven times under standardized conditions of light and darkness (Fig. 1). The right eye was always examined before the left. The subject kept the eye not being examined open, when the room was in light. The initial recording was done in standardized light (120 lux in the position of the eyes; Fig. 1). Then the subject looked with both eyes into a 55-W lamp for 5 minutes from a distance of 20 cm (1350 lux in the position of the eyes), after which the right and the left eyes were examined. Consequently, the left eye was exposed to light somewhat longer than the right eye before examination (Fig. 1). Then the monitor and the control panel were covered with red plastic film (Lee Filters, 027 medium red, BS 3944: Part 1, 1992; Light transmission: 0% [600 nm], 50% [650 nm], >50% [700 nm]). All leaks of light were eradicated. The examination room was in complete darkness except for the red light emitted from the monitor and control panel. The subject kept both eyes closed during the period of darkness. Measurements in the right and left eyes were done after 5, 15, and 25 minutes of darkness. The light in the room was then turned on, the red filter was removed, and the sixth measurement of flow velocities in the right and left eyes was performed. Finally, the subject with both eyes open was once again exposed to the light from the 55-W lamp for 5 minutes, whereafter the right and the left eyes were reexamined.

From the Departments of 1Ophthalmology, 2Clinical Physiology, and 3Neurology, University Hospital MAS, University of Lund, Malmö, Sweden.

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Proprietary interest category: N.

Reprint requests: Ulf Havelius, Department of Ophthalmology, University Hospital MAS, S-205 02 Malmö, Sweden.


The regression coefficients were calculated from registrations 2 to 5 for systolic and diastolic flow velocities and resistive index in the central retinal artery and the ophthalmic artery. The regression coefficients were tested against zero (hypothesis tested: b = 0).

The study was approved by the Ethics Committee at the University of Lund. The subjects gave informed consent to participate in the study. All experimental procedures conformed to the tenets of the Declaration of Helsinki.

**RESULTS**

**Ophthalmic Artery**

In the ophthalmic arteries the mean predarkness systolic velocities (range, 42.8–45.1 cm/s) tended to be higher than those in darkness (Fig. 2). The maximal reduction of systolic velocity in darkness was 5.6 cm/s (12%; \(P = 0.09\)) in the right eye and 5.0 cm/s (11%; \(P = 0.06\)) in the left eye. After 25 minutes in darkness the mean systolic velocities in the left eye showed an increase to the predarkness levels both immediately after the light was turned on and after 5 minutes in bright light (5.1 and 5.1 cm/s; \(P = 0.06\) and 0.02). The corresponding changes in the right eye were notably less marked and not significant.

The diastolic flow velocities in the ophthalmic arteries (range, 8.8–11.9 cm/s) did not consistently change from light to darkness. The resistive indices in the ophthalmic arteries did not show any conclusive changes.

**Central Retinal Artery**

During the light-darkness-light exposures (Fig. 1) there were marked changes in the systolic and diastolic flow velocities in the central retinal artery (Fig. 3). The mean systolic flow velocity, which was 7.4 to 7.7 cm/s in standard and bright light, increased with the duration in darkness and exceeded after 25 minutes the velocity in light by approximately 2 cm/s (range, 9.5–9.8 cm/s). This increase was highly significant on both sides as the differences were larger and more consistent in the right eye (15%; \(P = 0.002\)) and by 13% in the left eye (14%; \(P = 0.02\)). The mean decrease in flow velocity was slightly more pronounced after bright light, 1.7 cm/s on both sides (right eye: 17%; \(P = 0.002\); left eye: 18%; \(P = 0.0005\)).

Similar changes were seen in the diastolic flow velocities in darkness in the central retinal arteries (Fig. 3). The mean velocity in standard and bright light predarkness ranged between 1.9 and 2.2 cm/s. The increases reached statistical sig-
significance after 15 and 25 minutes in darkness compared with
standard light. The maximal increase was 1.3 cm/s in the right
eye after 25 minutes (68%; \( P < 0.001 \)) and in the left eye 1.0
cm/s after 15 minutes (53%; \( P = 0.001 \)). With re-exposure to
light there were no consistently significant changes.

The resistive index of the central retinal artery in the initial
standard light was compared with the values after 15 and 25
minutes in darkness. The resistive index in darkness showed a
trend toward reduction (Table 1).

The four consecutive registrations (after bright light and
after 5, 15, and 25 minutes in darkness) were also used to
calculate the regression coefficients for the changes in velocity
per minute. This was done for the systolic and diastolic veloc-
ities and the resistive index of the right and left ophthalmic
artery and central retinal artery. The regression coefficients
were tested against zero. For the central retinal artery the
differences were highly significant for the systolic velocity on
both sides (right eye: \( b = 0.074, P < 0.005 \); left eye: \( b = 0.068,
\ P < 0.005 \)). For the diastolic velocity there was also statistical
significance for both eyes (right eye: \( b = 0.034, P < 0.025 \); left
eye: \( b = 0.026, P < 0.05 \)). The other calculated differences
were nonsignificant.

**FIGURE 2.** The peak systolic and end-
diastolic flow velocities (mean ± SEM)
in the ophthalmic artery (OA) of the
right and left eyes of 12 healthy sub-
jects under different conditions of
light and darkness. The seven consec-
utive Doppler registrations (1-7) are
indicated on the abscissa. ●, right eye;
○, left eye.
DISCUSSION

This study was performed in a group of selected healthy subjects (nonsmokers), and the results should therefore reflect the physiological changes in ocular blood flow that occur in the transition from light to darkness and vice versa.

In humans information about ocular blood flow changes in darkness is sparse. In 1983 Feke et al. and Riva et al. each reported results from similar studies in three healthy subjects, examined by bidirectional fundus laser Doppler velocimetry. They used a helium-neon laser and reported increased retinal blood flow velocity after the transition from light to dark, interpreted as a consequence of an increased retinal oxygen consumption in darkness. However, one considerable disadvantage with the helium-neon laser Doppler velocimeter is that the laser beam has a light-adapting effect. Because blood flow velocity cannot be measured in complete darkness by this method it was assumed that the average velocity measured within
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TABLE 1. Comparison of Resistive Index (mean ± SEM) in the Central Retinal Artery in 12 Subjects

<table>
<thead>
<tr>
<th>Eye Examined</th>
<th>Standard Light</th>
<th>Darkness 15 Min</th>
<th>Darkness 25 Min</th>
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<tbody>
<tr>
<td>Right</td>
<td>0.74 ± 0.02</td>
<td>0.68 ± 0.02</td>
<td>0.68 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>0.76 ± 0.03</td>
<td>0.70 ± 0.02</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

15 to 20 seconds after turning on the laser beam would indicate blood velocity during darkness. Consequently, it may be concluded that the helium–neon laser is not ideal for examining the effects of light and dark on the retinal circulation.

To avoid this problem, in 1987 Riva et al. used near infrared laser Doppler velocimetry in a single subject to examine retinal blood velocity in darkness. Surprisingly, in this individual the blood velocity decreased slightly during darkness, although not significantly. After illumination there was a rapid (within 10–20 seconds) and marked increase in retinal blood flow velocity. They concluded that blood velocity does not increase in darkness and that the sudden transition from dark to light caused the previously observed increase rather than the prolonged state of dark adaptation.

In the present study we used CDI. There are certain advantages with CDI compared with laser Doppler velocimetry when examining ocular flow velocity in light and darkness. The problem that the laser beam causes (illumination of the retina) is avoided. The examination can be performed in complete darkness with the subject’s eyes closed (with the weak light sources in the room covered by a red filter). Repeated examinations can be done. There is no need for strict visual fixation by the subject. One disadvantage with the CDI technique may be the possibility of increasing IOP by the gentle pressure of the probe on the globe. This influence is minimized when the examination is performed by an experienced ultrasonographer.

Although there were no significant changes in the blood flow velocities in the ophthalmic artery between light and darkness, there may be a trend toward a lower systolic flow velocity in darkness (Fig. 2). The reason for this may be that according to the normal vascular anatomy of the orbita, the flow velocity in the ophthalmic artery was measured distal to the branching off of the central retinal artery. Furthermore, the ophthalmic artery has a larger lumen diameter and considerably higher systolic and diastolic flow velocities than the central retinal artery. Consequently, even marked changes in the flow in the central retinal artery (see below) may not be accompanied by statistically significant flow velocity changes in the ophthalmic artery. It may be added that the reduced choroidal flow in darkness, secondary to a reduced need for retinal “cooling,” may contribute to a lowered flow velocity in the ophthalmic artery.

The main finding in this study was that the systolic and diastolic blood flow velocities in the central retinal artery were markedly increased in darkness and that the systolic velocity was reduced after re-exposure to light (Fig. 3). Somewhat unexpectedly, the diastolic velocities in both eyes did not return to baseline values after re-exposure to light. This may reflect a rather slow desadaptation process, for which the time course of the possibly accompanying change in flow is unknown.

What may be the reason for the increase in flow velocity in the central retinal artery in darkness? Factors of relevance for flow velocity are the mean arterial blood pressure, IOP, blood viscosity, lumen diameter of the vessel, and peripheral resistance.

An increased mean arterial blood pressure in darkness is theoretically a possible explanation. However, the mean arterial blood pressures in supine position in six healthy individuals did not change significantly (authors’ unpublished observations) during dark adaptation compared with light adaptation (see “Methods”). Furthermore, in 1991 Guthoff et al. did not find any correlation between the systolic flow velocity in the central retinal artery and the systolic brachial artery blood pressure in a group of normal individuals. Consequently, the observed changes in flow velocities can hardly be ascribed to variations in systemic blood pressure.

Another possible explanation for the increase in flow velocity in the central retinal artery in darkness might be a decrease in IOP. All subjects in this study had normal IOPs. It is known that normally there is an increase in IOP amounting to a few millimeters of mercury in darkness. This increase may be due to changes in vasoregulation, but it is not caused by any change of the pupillary diameter. It is also known that artificial elevation of IOP by suction cup dynamometry in normal eyes will result in progressive diminution of velocity with increasing IOP. However, in normal eyes Guthoff et al. (1991) found no correlation between the systolic flow velocity in the central retinal artery and the IOP. Consequently, our finding of an increased flow velocity in the central retinal artery in darkness cannot be explained by any expected change in the IOP in darkness.

Among the remaining factors governing flow velocity, changes in viscosity should reasonably be excluded, considering the short duration of the experiments.

Changes in the lumen diameter of the examined vessel affect blood flow velocity, provided that all other parameters (e.g., perfusion pressure, blood viscosity) remain constant. The possible variation, between light and darkness in the lumen caliber of the central retinal artery at the position of repeated color Doppler measurements, is not known. Information about changes in the caliber of the retinal arteries in light and darkness is sparse in the literature. Feke et al. reported a negligible dilatation of larger branch retinal arteries in humans, amounting to 2% to 3% in darkness, whereas Hill and Housman did not find any conclusive changes in the caliber of retinal arterioles between light and darkness in the cat.

Assuming that luminal changes in the central retinal artery between light and darkness are minor, the only possible interpretation of our findings is that there is a decrease in peripheral retinal vascular resistance in darkness. The fall in resistive index (Table 1), derived to reflect variations in the peripheral vascular resistance, supports such an interpretation. Furthermore, Feke et al. and Riva et al. reported an increase in the diameter of large retinal veins, amounting to 5% to 8% after 20 to 30 minutes of dark adaptation, a finding that may be compatible with such an interpretation.

Having considered and excluded various other possible explanations, the progressive increases in systolic and diastolic flow velocities in the central retinal artery with time spent in darkness and subsequently improving dark vision...
(Fig. 3) may possibly reflect an increased metabolic demand by the photoreceptors. Experimental studies in animals have shown that darkness is accompanied by a markedly increased oxidative metabolism in the retinal photoreceptors.1–3 Consequently, dark vision is critically dependent on a sufficient oxygen supply.

What may then be the explanation for the increase in flow velocity in the central retinal artery? The retina has a dual vascular supply: the retinal arteries and the choroidal circulation. The contribution of oxygen from each of these sources at different retinal depths depends on conditions of light and darkness.2,3 The oxygen derived from the choroid reaches the inner segments of the photoreceptors independent of light conditions. In darkness the photoreceptors have a markedly raised oxidative metabolism, and, consequently, the amount of oxygen diffusing from the choroid into the retina beyond the photoreceptors is reduced. Because the inner retina maintains its oxidative metabolism irrespective of light and dark,1–3 the oxygen tension in the inner retina will fall in darkness and trigger an increased retinal blood flow by mechanisms of autoregulation.

Acknowledgments

The authors thank Anette Holmén and Elzbieta Krolikiwska, Department of Clinical Physiology, University Hospital, Malmö, Sweden, for performing the Doppler measurements.

References


Nε-(Carboxymethyl)Lysin and the AGE Receptor RAGE Colocalize in Age-Related Macular Degeneration

Hans-Peter Hammes,1 Hans Hoerauf,2 Alex Alt,1 Erwin Schleicher,3 Jes Thorn Clausen,4 Reinhard G. Bretzel,1 and Horst Laqua2

PURPOSE. To investigate whether glycoxidation products and the receptor for advanced glycation end products (RAGE) are present in subfoveal membranes of patients with age-related macular degeneration (ARMD).

METHODS. Surgically removed subfoveal fibrovascular membranes from 12 patients, 11 related to ARMD and 1 to an idiopathic membrane, were analyzed for the presence of the glycoxidation product Nε-(carboxymethyl)lysine (CML), one of the receptors for advanced glycation end products, RAGE, and the activation of NFkB, using immunochemistry.

RESULTS. CML-like immunoreactivity was found in all ARMD specimens examined adjacent or colocalized with RAGE, but not in the idiopathic membrane. RAGE immunoreactive material was found in CD68-positive cells and in the fibrous matrix. CD68-positive cells and surrounding areas stained for p50, the activated form of NFkB.

CONCLUSIONS. These results indicate that glycoxidation products are present in subretinal membranes of patients with ARMD. The concomitant expression of RAGE in these membranes and the finding of activated NFkB is suggestive of an implication of glycoxidation product formation in the pathogenesis of the disease. (Invest Ophthalmol Vis Sci. 1999;40:1855–1859)

Age-related macular degeneration (ARMD) is the main cause of legal blindness in the elderly population.1 Advanced stages of ARMD develop into two variants, one of which involves sprouting of new blood vessels from the choriocapillaris into the subretinal space.2 Despite detailed knowledge about the natural course of choroidal neovascularizations, the underlying cause remains poorly understood.

The aging process is associated with increased formation and deposition of chemically modified proteins, lipids, and nuclear acids called advanced glycation end products (AGE; for
products form by sequential glycation and oxidation (glycoxidation products), such as Nε-(carboxymethyl) lysine (CML), which is not only a glycoxidation product, but can also form from the peroxidation of lipoproteins.

Glucose-modified bovine serum albumin (BSA) containing CML has been shown to induce the expression of growth factors in vitro, thus linking the CML and other AGE with the formation of neovascularizations in aged patients. We speculated that CML could be involved in the formation of subretinal neovascular membranes, and we investigated whether CML is present in subretinal membranes of patients with ARMD and studied the presence and localization relative to CML of one of the AGE receptors, RAGE, in these membranes.

**MATERIALS AND METHODS**

For the human research, the tenets of the Declaration of Helsinki were followed, and informed consent was obtained.

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**FIGURE 1.** Immunolocalization of CML-modified proteins in subfoveal neovascular membranes from patients with ARMD. (A) Negative control of a membrane showing fibrovascular (white arrow) and fibroproliferative (black arrow) segments, and areas of foamlike deposits (white arrowhead). (B) Section adjacent to (A) stained with the polyclonal CML antibody. Note the strong immunolabeling of fibrovascular and fibroproliferative areas and near the foamlike deposits. *Gray arrow* indicates area used for colocalization studies shown in Figure 2. CML-positive deposits in (C). Areas of cellular infiltrations in (D) matrix, (E) in fibrillous deposits, and (F) amorphous deposits. (G) CML staining of the retina. (H) RAGE staining of the retina. (C, D, E, F, G) Monoclonal CML antibody (6D12). Original magnification, (A, B) ×63; (C, D, E, F, G) ×25.

**FIGURE 2.** Accumulation of CML in areas of subfoveal membranes colocalizing with CD68+ cells expressing RAGE and activated NFkB p50. (A) Magnification ×25 of the area indicated in Figures 1A and 1B. (gray arrow), stained with the polyclonal CML antibody. (B) Adjacent section stained with the polyclonal RAGE antibody. (C) Adjacent section stained with the monoclonal CD68 antibody, labeling macrophages. (D) Adjacent section stained with the polyclonal antibody against NFkB p50. (E) Negative control of (A), incubated with nonimmune IgG. Black arrows indicate corresponding areas.
Materials

Twelve subfoveal neovascular membranes, 11 excised from patients with age-related macular degeneration (aged 72–81 years, 10 with occult and 1 with a classic subfoveal neovascular membrane) and 1 excised from a patient with idiopathic neovascular membrane (female, age 17) were examined after removal during a three-port pars plana vitrectomy. Membranes were immediately placed in Bouin fixative for 12 hours at room temperature and subsequently embedded in paraffin. Serial sections were cut at 5 µm. One donor eye was obtained from a 42-year old male donor without diabetes or any known ocular disease. After fixation in Bouin fixative for 12 hours, the retina was similarly processed for immunohistochemistry.

Immunohistochemistry

CML. Paraffin-embedded subretinal membrane sections were immunostained using a standard alkaline phosphatase–anti-alkaline phosphatase protocol and a monoclonal anti-CML antibody (6D12) whose specificity has been described previously.\(^1\) Histochemical detection was performed using newfuchsian chromogen substrate (Dako; Carpinteria, CA) as chromogen and hemalaun as a counterstain. For control sections, the primary antibody was omitted or replaced by a nonimmune mouse IgG. Additional sections were stained using a polyclonal antibody recognizing protein-bound CML, which was characterized previously, and the avidin-biotin, complex peroxidase staining method (ABC; Vector, Burlingame, CA) as described.\(^5\)

RAGE. RAGE immunohistochemistry was performed using a peroxidase-antiperoxidase protocol and a polyclonal antibody raised against the extracellular part of the RAGE (truncated RAGE). The gene sequence for the extracellular part of the human RAGE described by Schmidt et al.\(^6\) was obtained from Gene Bank. cDNA was cloned from a human lung cDNA library by standard reverse transcription–polymerase chain reaction procedures, inserted into the vector pBac, and expressed in the baculovirus system. New Zealand White rabbits were injected subcutaneously with 50 µg of the truncated RAGE emulsified in Freund’s complete adjuvant, followed by three booster injections with 50 µg truncated RAGE emulsified in Freund’s incomplete adjuvant every 2 weeks. Ten days after the last injection, the animals were bled, and sera were isolated. The antibody was characterized by western blot analysis using membranes from Hek 293 cells transfected with RAGE and retinal extracts of diabetic and nondiabetic rats. A single band was observed in retinal extracts from normal and diabetic rats at M, of approximately 39 kDa, and bands with M, between 45 and 52 kDa were observed in samples from transfected cell membranes (because of different posttranslational glycosylation of RAGE in Hek cells), whereas no bands were detected in nontransfected cells. As controls, the antibody was preadsorbed with excess soluble RAGE or omitted. Both cases resulted in the disappearance of the band (not shown). Antibody binding was visualized by 3,3’-diaminobenzidine (0.06%), and sections were counterstained with hemalaun.

Semiquantitative Grading of CML and RAGE Immunoreactivity

Staining intensity of the monoclonal CML antibody was evaluated using a four-step system (0, unreactive; +, mildly positive; ++, moderately positive; and ++++, strongly positive). The grading was performed for areas with high cellularity and for matrix-rich areas of the membranes by two observers unaware of the samples’ identity (HPH, AA).

NFkB p50

To identify the activated transcription factor NFkB p50 in subretinal membranes, sections were incubated with an affinity-purified rabbit polyclonal antibody (2 µg/ml) raised against amino acids 350 to 363 mapping within the nuclear location signal region of human NFkB 50 (Santa Cruz Biotechnology, Santa Cruz, CA), and detection was performed using the indirect immunoperoxidase method (ABC Kit, Vector).

CD68

For the identification of human macrophages, sections were immunostained with an anti-human CD68 monoclonal antibody (4 µg/ml; Dako) and the alkaline phosphatase detection system as described.

Results

Subretinal neovascular membranes consisted of areas with fibrovascular and fibrous tissue, focal RPE deposits, depositions of amorphous material, and focal cell infiltrations (Fig. 1A). CML-immunoreactive material was found in all membranes examined and was heterogeneously distributed within the membranes, including fibrovascular and fibrous elements, foamlike deposits, and near RPE inclusions (Fig. 1B).

Using the monoclonal antibody, CML was detected in the vicinity of vascular structures (Fig. 1C), in perinuclear cell compartments, in the extracellular matrix (Fig. 1D), in foamlike areas (Fig. 1E), and in amorphous material (Fig. 1F). In the retina of a patient without known ocular diseases, no CML reactivity was discernible (Fig. 1G), but RAGE was moderately present (Fig. 1H). The CML-immunoreactive material was strongly positive (Fig. 1I), whereas RAGE was moderately positive (Fig. 1J) in the retinal extracts from diabetic and nondiabetic rats. A single band was observed in retinal extracts from normal and diabetic rats at M, of approximately 39 kDa, and bands with M, between 45 and 52 kDa were observed in samples from transfected cell membranes (because of different posttranslational glycosylation of RAGE in Hek cells), whereas no bands were detected in nontransfected cells. As controls, the antibody was preadsorbed with excess soluble RAGE or omitted. Both cases resulted in the disappearance of the band (not shown). Antibody binding was visualized by 3,3’-diaminobenzidine (0.06%), and sections were counterstained with hemalaun.

Table 1. Semiquantitative Evaluation of CML Immunohistochemistry in Subfoveal Neovascular Membranes of Patients with ARMD (Cases 1–11) and in One Patient (Case 12) with Idiopathic Subfoveal Neovascular Membrane

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<tbody>
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<td>1</td>
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</tr>
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<td>+++</td>
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<td>+++</td>
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<td>+++</td>
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<td>0</td>
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</tr>
<tr>
<td>6</td>
<td>+*</td>
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<td>12</td>
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Grading is according to a four-step system: 0, unreactive; +, mildly positive; ++, moderately positive; ++++, strongly positive. The grading system is described in the Methods section.

* Membranes very small; no cellular structures discernible.
present in the inner limiting membrane and the inner plexiform and nuclear layers (Fig. 1H).

To study the possible colocalization of CML with RAGE, we used defined fibrovascular areas (Figs. 1A, 1B and Figs. 2A, 2B, 2C, 2D, 2E). When compared with the immunolabeling of CML (Fig. 2A), RAGE colocalized in fibrovascular areas of the membranes and near RPE deposits (Fig. 2B). These findings were consistent for all samples examined (for quantitation see Table 1). The distribution of CML- and RAGE-immunoreactivity indicate a colocalization of both in neovascular membranes of patients with ARMD.

Macrophages are one cell type expressing RAGE, indicated by CD68 immunostaining in areas of RAGE immunostaining (Figs. 2B, 2C).

Next, we hypothesized that cells expressing RAGE would show functional consequences induced by binding of AGE-type ligands to RAGE. It was found that the activated form of NFκB, NFκB p50, was widely expressed by cells in these membranes, indicating the functional role of RAGE in the subretinal membranes of ARMD patients (Figs. 2D).

In contrast to these findings, and to demonstrate that ARMD is different from other diseases associated with subretinal neovascularizations, we used the same histochemical parameters to study the subretinal membrane from a 17-year-old girl with idiopathic subretinal neovascular membrane. This membrane was negative for CML and RAGE (Figs. 3A, 3B).

**DISCUSSION**

Our findings indicate that CML-like immunoreactive material is present in membranes of patients with ARMD, RAGE is associated with CML-immunoreactive material within membranes, RAGE is expressed by CD68-positive cells in this tissue, and NFκB p50 is present as an indication of functional activation.

CML is involved in the aging process, because it accumulates in long-lived matrix structures such as skin collagen of normal people. Although CML neither forms crosslinks nor induces free radicals, it is regarded as a biomarker of oxidant stress in the respective tissues. CML is absent from tissues of young age and is not found in retinas from control subjects, at least not with the CML antibody used in the present study. The presence of CML in subfoveal neovascular membranes is surprising, because the material was not deposited long enough to assume CML accumulation with age. It is more likely, that CML accumulation, or an underlying process leading to it, is involved in the pathogenesis of subretinal neovascularization.

CML is colocalized in the subretinal membranes with RAGE. On binding to AGE, RAGE mediates a variety of cellular responses, including increased intracellular oxidant stress and chemotaxis. Preliminary data indicate that CML-modified proteins, as recognized by the antibodies used in our study, are ligands for RAGE, activating intracellular signaling pathways and altering gene expression. Moreover, reactive oxygen intermediates themselves can stimulate RAGE expression because reactive oxygen intermediates induce NfκB, and the RAGE promoter has two NfκB-binding sites.

Subretinal neovascularization associated with ARMD can be explained, at least in part, by the activity of factors stimulating the growth of both vascular and matrix components. Among others, basic fibroblast growth factor and vascular endothelial growth factor (VEGF) have been found in subfoveal fibrovascular membranes. Whereas the exact biochemical stimulus for basic fibroblast growth factor is unknown, the major stimulus for VEGF expression is hypoxia. VEGF stimulation in ARMD may be the result of increased oxidative stress, and the formation of glycoxidation products may be a contributing factor. Both reactive oxygen intermediates and AGE are capable of inducing VEGF expression in retinal cells, especially in RPE, and AGES by themselves are angiogenic.

The scenario of CML colocalized with RAGE and inflammatory cells present in neovascular membranes suggests that this system may be involved in the initiation and/or propagation of ARMD. Supporting evidence for our observations comes from a recent study showing that CML is present in soft drusen (preceding choroidal neovascular membranes) and in nearby RPE cells, whereas in control eyes, no CML was found. The role of RAGE was not investigated.

Taken together, the evidence in this study suggests that CML-like immunoreactivity is present in subfoveal neovascular membranes of patients with ARMD and that they colocalize with one of the AGE receptors, RAGE. Because current therapeutic options are limited, further studies are needed to determine the implication of these findings and their functional consequences in the pathogenesis of ARMD. More detailed studies extending this and other preliminary observations, are warranted.

**Acknowledgments**

The authors thank Dres Henrik Vissing and Poul Baad Rasmussen, Novo Nordisc, for cloning and production of the AGE-receptor; Seikoh Horiiuchi for his kind gift of the 6D12 monoclonal CML-antibody; and Kerstin Schneider for help with the manuscript.

**References**

2. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-
Frequency of Mutations in the Gene Encoding the $\alpha$ Subunit of Rod cGMP-Phosphodiesterase in Autosomal Recessive Retinitis Pigmentosa

Thaddeus P. Dryja,1 David E. Rucinski,1 Sherileen Huang Chen,1 and Eliot L. Berson2

PURPOSE. To determine the mutation spectrum of the PDE6A gene encoding the $\alpha$ subunit of rod cyclic guanosine monophosphate (cGMP)-phosphodiesterase and the proportion of patients with recessive retinitis pigmentosa (RP) due to mutations in this gene.

METHODS. The single-strand conformation polymorphism (SSCP) technique and a direct genomic sequencing technique were used to screen all 22 exons of this gene for mutations in 164 unrelated patients with recessive or isolater RP. Variant DNA fragments revealed by SSCP analysis were subsequently sequenced. Selected alleles that altered the coding region or intron splice sites were evaluated further through segregation analysis in the families of the index cases.

RESULTS. Four new families were identified with five novel mutations in this gene that cosegregated with disease.

Concluding. The PDE6A gene appears to account for roughly 3% to 4% of families with recessive RP in North America. A compilation of the pathogenic mutations in PDE6A and those reported in the homologous gene PDE6B encoding the $\beta$ subunit of rod cGMP-phosphodiesterase shows that the cGMP-binding and catalytic domains are frequently affected. (Invest Ophtalmol Vis Sci. 1999;40:1859–1865)

Retinitis pigmentosa (RP) is a hereditary degenerative disease of the retina leading to blindness. There are both syndromic and nonsyndromic forms, and the inheritance can be dominant, recessive, X-linked, maternal, or digenic. It is genetically heterogeneous with more than 50 loci implicated in the pathogenesis and in sequence.2–4 Mutations in the PDE6B gene (chromosome 4p16.3)5,6 encoding the $\beta$ subunit of rod cGMP-phosphodiesterase account for approximately 4% of cases of recessive RP.5–8 With regard to the PDE6A gene (chromosome 5q31.2)2 encoding the $\alpha$ subunit, our group9 and Meins et al.3 have described mutations in only three families with recessive RP. The report from our group was a partial evaluation of some of the exons of this gene.9 Here, we describe a comprehensive evaluation of all 22 exons in a subset of the same group of patients.

METHODS

This study, which involved human subjects, conformed to the tenants of the Declaration of Helsinki. The diagnostic
criteria used at this center for RP and for a recessive inheritance pattern have been described previously.\textsuperscript{10,11} We excluded at the outset patients with RP known to be caused by pathogenic mutations in any other RP gene that had been analyzed in our laboratory. All the patients in this set had been included in our previously published evaluation of some of the exons of the \textit{PDE6A} gene.\textsuperscript{9} That report involved 173 patients with recessive RP, 9 of whom were excluded from this study. Patients were excluded because a pathogenic mutation in another RP gene has since been discovered, because a review of records uncovered that two index cases from the same family were both inadvertently included previously as unrelated patients, or because there was insufficient leukocyte DNA available in the laboratory to complete this study. Some patients previously misclassified as having recessive RP were recategorized as having isolate RP, because they had no affected siblings, and they were not the offspring of a consanguineous marriage. One hundred sixty-four patients were in this study, comprising 146 patients with recessive RP and 18 newly recategorized patients with isolate RP. Most of the patients resided in the United States or Canada and, as a group, were of ethnic composition comparable to the affected patients in this study.

The laboratory analysis was performed on DNA purified from blood samples from these patients. Mutations were discovered using the single-strand conformation polymorphism (SSCP) technique.\textsuperscript{12} Primer sequences and reaction conditions for amplifying each of 22 exons were identical with those reported previously by Huang et al.,\textsuperscript{9} except for exons 6 and 13, for which the following pairs of primers were used, respectively (sense; antisense): 5'-ATTTTTCTCTTTGCGAAG-3'; 5'-TTCTGCTTTGACAGGTGAAAC-3'; 5'-TATTCCAACCCTCATGAGAC-3'; 5'-TACCATGTAGAGTCTGCATG-3'. This analysis included the entire coding sequence and flanking intron sequences containing all splice donor and acceptor sites except the splice acceptor site of intron 1. DNA samples with variant bands observed by SSCP were subsequently sequenced. The investigation of the segregation of alleles was through analysis by SSCP or by direct sequencing of index patients and all available relatives.

Computations of the likelihood that a sequence variant might create a splice-acceptor or splice-donor site were according to the method of Reese et al.\textsuperscript{13} and were performed by that
RESULTS

We discovered five novel mutations that were judged to be responsible for recessive RP. Using published recommendations for naming mutations,14 these mutations are Arg102His (CGC to CAC), Arg102Ser (CGC to AGC), IVS6+1G→A, Gln569Lys (CAG to AAG), and Ser573Pro (TCC to CCC) (Fig. 1). Each of these changes was found in affected subjects who were either homozygotes or compound heterozygotes for mutations in this category. In addition, these mutations cosegregated with RP in the respective families (Fig. 2). None of them was found among unrelated normal control subjects (70 control subjects were evaluated for the mutations Arg102His and Arg102Ser in exon 1, 92 for the mutation at the splice-donor site of intron 6, and 79 for the mutations Gln569Lys and Ser573Pro in exon 13.)

Six missense changes of uncertain pathogenicity were identified: Asn216Ser, Val277Ala, Pro293Leu, Val391Met, Lys827Gln, and Gly850Val (Table 1). None of these appeared to be a polymorphism, because the minor allele frequencies were less than 1% among the set of surveyed patients. Each was found heterozygously in only one or two unrelated patients. No abnormality was detected in the PDE6A sequence of the other allele in any of these patients. The Val277Ala change was not found in the only affected sibling of the index patient, and it is therefore not likely to be the cause of recessive RP in that family. The Pro293Leu change was present heterozygously in the only affected child of parents who were first cousins; because a homozygous mutation would be expected in the affected offspring from consanguineous parents, the allele is also unlikely to be the cause of recessive RP. However, these results do not rule out the possibility that the Val277Ala and Pro293Leu changes are pathogenic, only that they are not responsible for RP in these families. The Asn216Ser and Lys827Gln changes were present heterozygously in the affected siblings of the index cases with recessive RP, but in each case there was only one affected sibling; therefore, this cosegregation could be explained by chance alone. The family of the index patient with the Val391Met change was unavailable for segregation analysis. The segregation analysis of the Gly850Val change was uninformative: the change was present heterozygously in one patient in the survey of Meins et al.3 (It was labeled Gly849Val in that article; it is renumbered as Gly850Val here because of a revision in the cDNA sequence found in Table 3 of Meins et al.3 that changes the specificity and numbering of codons after 845.)

We encountered 17 variant sequences that were judged unlikely to affect the sequence of the encoded protein (Table
### Table 1. Polymorphisms, Rare Silent Variants, and Rare Variants of Uncertain Pathogenicity

<table>
<thead>
<tr>
<th>Sequence Variation</th>
<th>Exon or Intron</th>
<th>Recessive RP</th>
<th>Isolate RP</th>
<th>Normal Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr<em>28Tyr</em> (TAC vs. TAT)</td>
<td>1</td>
<td>290:2</td>
<td>36:0</td>
<td></td>
</tr>
<tr>
<td>Ser<em>34Ser</em> (TCC vs. TCT)</td>
<td>1</td>
<td>291:1</td>
<td>35:1</td>
<td>140:0</td>
</tr>
<tr>
<td>Arg<em>11Arg</em> (AGG vs. CGG)</td>
<td>1</td>
<td>256:36 (0.123)</td>
<td>29:7 (0.194)</td>
<td>126:14 (0.100)</td>
</tr>
<tr>
<td>Asn<em>155Asn</em> (AAC vs. AAT)</td>
<td>1</td>
<td>256:36 (0.123)</td>
<td>29:7 (0.194)</td>
<td>126:14 (0.100)</td>
</tr>
<tr>
<td>IVS2 – 68 T vs. C</td>
<td>IVS2</td>
<td>288:4 (0.014)</td>
<td>36:0 (0.000)</td>
<td>ND</td>
</tr>
<tr>
<td>Asn<em>216Ser</em> (AAT vs. AGT)</td>
<td>3</td>
<td>291:1</td>
<td>35:1</td>
<td>ND</td>
</tr>
<tr>
<td>IVS3 – 4 insertion T</td>
<td>IVS3</td>
<td>291:1</td>
<td>35:1</td>
<td>ND</td>
</tr>
<tr>
<td>Val<em>277Ala</em> (GGG vs. GTG)</td>
<td>4</td>
<td>289:2</td>
<td>36:0</td>
<td>184:0</td>
</tr>
<tr>
<td>IVS4 – 63 (A vs. G)</td>
<td>IVS4</td>
<td>271:21 (0.072)</td>
<td>31:5 (0.139)</td>
<td>ND</td>
</tr>
<tr>
<td>Pro<em>293Leu</em> (CCG vs. CTG)</td>
<td>5</td>
<td>291:1</td>
<td>35:1</td>
<td>ND</td>
</tr>
<tr>
<td>IVS6 + 11 C vs. T</td>
<td>IVS6</td>
<td>291:1</td>
<td>36:0</td>
<td>ND</td>
</tr>
<tr>
<td>Ala<em>362Ala</em> (GCG vs. GCA)</td>
<td>8</td>
<td>291:1</td>
<td>36:0</td>
<td>ND</td>
</tr>
<tr>
<td>Val<em>391Met</em> (GTT vs. ATG)</td>
<td>9</td>
<td>285:7 (0.024)</td>
<td>35:1 (0.028)</td>
<td>ND</td>
</tr>
<tr>
<td>IVS10 – 34 A vs. G</td>
<td>IVS10</td>
<td>291:1</td>
<td>36:0</td>
<td>ND</td>
</tr>
<tr>
<td>IVS10 – 26 insertion T</td>
<td>IVS10</td>
<td>291:1</td>
<td>36:0</td>
<td>ND</td>
</tr>
<tr>
<td>Phe<em>597Phe</em> (TTC vs. TTC)</td>
<td>14</td>
<td>279:13 (0.045)</td>
<td>31:5 (0.139)</td>
<td>ND</td>
</tr>
<tr>
<td>IVS15 – 21 C vs. G</td>
<td>IVS15</td>
<td>288:4 (0.014)</td>
<td>35:1 (0.028)</td>
<td>ND</td>
</tr>
<tr>
<td>IVS18 + 21 A vs. C</td>
<td>IVS18</td>
<td>281:11 (0.038)</td>
<td>31:5 (0.139)</td>
<td>184:6 (0.032)</td>
</tr>
<tr>
<td>Gly<em>465Gly</em> (GGT vs. GGG)</td>
<td>19</td>
<td>291:1</td>
<td>36:0</td>
<td>ND</td>
</tr>
<tr>
<td>Phe<em>779Phe</em> (TCT vs. TTC)</td>
<td>20</td>
<td>278:14 (0.047)</td>
<td>31:5 (0.139)</td>
<td>ND</td>
</tr>
<tr>
<td>Asp<em>800Asp</em> (GAC vs. GAT)</td>
<td>21</td>
<td>281:11 (0.037)</td>
<td>31:5 (0.139)</td>
<td>ND</td>
</tr>
<tr>
<td>Gly<em>808Glu</em> (GAG vs. GAA)</td>
<td>21</td>
<td>287:5 (0.017)</td>
<td>36:0 (0.000)</td>
<td>ND</td>
</tr>
<tr>
<td>Lys<em>827Gln</em> (AAG vs. CAG)</td>
<td>21</td>
<td>291:1</td>
<td>36:0</td>
<td>ND</td>
</tr>
<tr>
<td>Gly<em>850Val</em> (GTA vs. GTC)</td>
<td>22</td>
<td>292:0</td>
<td>35:1</td>
<td>ND</td>
</tr>
</tbody>
</table>

+ Positively charged amino acid residue; − negatively charged amino acid residue; o, nonpolar amino acid residue; *, polar amino acid residue; ND, not done; IVS, intron. For the variants listed in the left column, the three columns on the right provide the number of alleles with the more common vs. the less common sequence found among 146 patients with recessive RP, 18 patients with isolate RP, and 70–95 normal controls. Minor allele frequencies are also provided in parentheses for polymorphisms (i.e., those variations with a minor allele frequency greater than 0.01.)

### DISCUSSION

Summing the results from this study and an earlier one performed by our group, eight pathogenic mutations in the PDE6A gene in 6 families were discovered among 164 families with recessive or isolate RP. From these results it can be estimated that approximately 3% to 4% of cases of recessive RP are caused by mutations in PDE6A. This value is approximate because of the small number of ascertained cases, because the SSCP screening technique misses approximately 10% of point mutations,13 because the SSCP technique usually does not detect large gene deletions or rearrangements, and because the upstream and downstream sequences of the gene and most of the intron sequence were not evaluated. The estimate of preva-
alence does not change substantially if the 13 patients with recessive RP who were excluded from this analysis are taken into account. (Those patients were excluded because they had mutations in other RP genes discovered before the onset of this study or during its course. Four of the excluded patients had recessive RP due to mutations in \textit{PDE6B}, six had mutations in the gene encoding the \textit{a} subunit of the cGMP-gated channel, one had a homozygous mutation in the rhodopsin gene, two had mutations in the \textit{TULP1} gene, and three had mutations in the \textit{RPE65} gene.) The estimated prevalence of 3% to 4% for \textit{PDE6A} mutations among families with recessive RP is close to the 4% prevalence estimated for \textit{PDE6B} by summing data from three groups (4 of 92 families, 6 of 19 families, and 2 of 101 families).

Of the two active subunits of rod cGMP-phosphodiesterase, the \textit{b} subunit is more often studied. Naturally arising, recessive defects in this gene cause retinal degeneration in mice (the \textit{rd} strain), Irish setter dogs, and humans. A dominant mutation in the \textit{PDE6B} gene has been discovered in some Danish families with congenital stationary night blindness. Mutations in the \textit{PDE6A} gene encoding the \textit{a} subunit cause recessive retinal degeneration in Welsh Cardigan Corgi dogs and recessive RP in humans.

The amino acids normally specified by codons 102, 569, and 573 affected by the novel missense changes described in this article are conserved among the following phosphodiesterase subunits: the \textit{a} subunit of mouse, cow, and dog; the \textit{b} subunit of human, cow, and dog; and the \textit{a} subunit of human, cow, and chick. Specifically, the positions equivalent to codons 102 and 569 are always Arg and Gln, respectively, whereas the position equivalent to codon 573 is either Ser or Thr, two residues with similar side groups. In contrast, most of the missense changes of uncertain pathogenicity affected residues that have not been highly conserved in evolution: the residue in the location of Asn216 is Ser in bovine cone phosphodiesterase, Val277 is Ile in human and bovine cone phosphodiesterase, Pro293 is Ser in human rod phosphodiesterase, and Lys827 and Gly850 are in a poorly conserved region near the carboxyl-terminus of the protein. The position equivalent to codon 391 is Val in all vertebrate rod and cone cGMP-phosphodiesterase \textit{a}, \textit{b}, and \textit{a} subunits sequenced to date. However, the Val391Met change would not substantially alter the side group at position 391, because both Val and Met have nonpolar side groups of similar size. These comparisons to photoreceptor phosphodiesterases from other vertebrates are weak evidence that the missense changes Asn216Ser, Val277Ala, Pro293Leu, Val807Met, and Gly850Val are not pathogenic. However, the evidence from these comparisons and from the

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933583/)
The mechanisms by which PDE6A and PDE6B mutations lead to RP are probably similar. Both the α and β subunits of cGMP-phosphodiesterase are necessary for the enzyme’s function. Mice and dogs with recessive mutations in the PDE6B gene have abnormally high concentrations of cGMP in their retinas before the severe loss of their photoreceptors.\(^4\)\(^5\) These elevated levels of cGMP arise presumably because of an absence of activity of cGMP-phosphodiesterase in the mutant photoreceptor cells. The cGMP levels are sufficiently elevated to be toxic to photoreceptors, although the specific biochemical pathways mediating the toxicity are unknown. One attractive hypothesis is that the high cGMP concentration causes an increase in the proportion of open cGMP-gated channels in the rod outer segment membrane. Only a small percentage of these channels are open in normal rod photoreceptors in the dark-adapted state when the cGMP concentration in the outer segment is physiologically at its highest level.\(^6\) The cGMP levels in mutant photoreceptors without functional phosphodiesterase are much higher than these physiologic concentrations. The high cGMP levels should result in a higher-than-normal proportion of open channels and a presumably toxic increase in the influx of sodium and calcium ions into the cytoplasm.

Most of the patients in this study had been evaluated for mutations in the PDE6B gene.\(^6\) We considered the possibility that some patients may have RP because of double heterozygosity for mutations in both the PDE6A and PDE6B genes. However, none of our patients with PDE6A alleles categorized as pathogenic also had a missense change in the PDE6B gene. This was also true for the patients with the rare variant missense changes in PDE6A of uncertain pathogenicity. These negative results leave as an unanswered question what the phenotype of a double (PDE6A plus PDE6B) heterozygote would be. Because recessive mutations of both genes are known in dogs,\(^21\)\(^36\) the procedure to determine the double-heterozygote phenotype in that species should be straightforward, through appropriate breeding.

Figure 3 depicts the locations of the known pathogenic mutations in the PDE6A and PDE6B genes causing retinal degeneration in humans, mice, and dogs. Mutations causing recessive RP were included in this figure only if they have been reported in affected people who are homozygotes or compound heterozygotes. The figure also shows the location of the missense mutation found in some Danish families with dominantly inherited congenital stationary night blindness.\(^8\)\(^34\)\(^35\) Among the mutations causing recessive RP, some are highly likely to be null alleles, because they are splice-site mutations or they are frameshift or nonsense mutations that would truncate the carboxyl-terminus of the protein and eliminate most or all the catalytic domains. The novel splice site mutation (IVS6+1G→A) found in this study is included in this set of highly likely null alleles. Of the 12 missense mutations in these genes, 9 affect residues within the cGMP-binding and catalytic domains or the isoerythrin site, including the 4 novel missense changes in the PDE6A gene (Arg102His, Arg102Ser, Gln569Iys, and Ser573Pro) described in this study. The PDE6B mutation Leu854Val\(^6\) affecting the isoerythrin site at the C terminus of the protein suggests that this posttranslational modification of the β subunit also is essential to the enzyme’s function in photoreceptors. The fact that many pathogenic mutations affect the cGMP-binding and catalytic domains is in accord with the hypothesis that retinal degeneration results from interference with phosphodiesterase activity and not with some other function of this enzyme. However, when all pathogenic missense changes in both genes are included, there is no statistically significant clustering of the mutations in these domains, considering that these domains represent approximately 65% of the primary sequence of the protein.

Acknowledgments

The authors thank Terri McGee, Carol Weigel-DiFranco, and Peggy Rodriguez for helpful comments and assistance.

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Topography of Cone Electrophysiology in the Enhanced S Cone Syndrome

Michael F. Marmor,1 Fang Tan,1 Erich E. Sutter,2 and Marcus A. Bearse, Jr2

PURPOSE. To investigate the topography of cone electroretinographic (ERG) responses in the enhanced S cone syndrome (ESCS).

METHODS. A 19-year-old female with ESCS who was one of the original cases defining the syndrome was studied. Full-field, focal (Maculoscope) and multifocal (VERIS) ERGs were performed using white light. Multifocal ERG responses were also generated with red and blue stimuli and with a slow m-sequence to elicit off-responses. Results were analyzed by averaging data in rings at increasing eccentricity from the fovea and compared to data recorded identically from a normal subject.

RESULTS. The full-field ERG from this patient showed typical large slow photopic waveforms and was unchanged from recordings made 9 years earlier. The focal ERG showed signals of borderline low amplitude from the fovea with the multifocal ERG, the ESCS responses from the central macula had a relatively normal waveform, and those 9° to 20° from fixation showed the prolonged waveform that characterizes the full-field ERG. Responses were larger to blue light than red light in ESCS in both center and periphery. The central ESCS responses were relatively normal in timing to both red and blue light, whereas the peripheral ESCS responses were markedly delayed to both. Off-responses were seen in ESCS only near the foveal center.

CONCLUSIONS. The marked differences between central and peripheral ERG responses in ESCS suggest that there are different distributions of S, L, and M cones in these regions and that S cones may feed into different neural pathways in the center and periphery. It was postulated that in ESCS, S cones may partially replace L and M cones in the central macula and feed into the usual S cone pathways. In the periphery, however, there is little L and M cone b-wave activity in ESCS, and S cones may usurp both the space and neural pathways of the rods. (Invest Ophthalmol Vis Sci. 1999;40:1866–1873)

The enhanced S cone syndrome (ESCS) is a rare inherited retinal disorder characterized by an absence of rod function and by large-amplitude S cone–mediated electrical responses that dominate the photopic electroretinogram.1,2 It appears to be a relatively stationary abnormality with respect to rod and S-cone function, although cystic maculopathy may develop in some patients. Clinically the fundus shows yellowish retinal pigment epithelial lesions in the region of the retinal vascular arcades. ESCS may lie on a spectrum with patients described as having Goldmann–Favre disease who show extensive retinal pigment epithelial and retinal degeneration with low-amplitude electroretinographic (ERG) signals but otherwise similar ERG characteristics.

Although several papers have demonstrated S cone spectral sensitivity for the conventional ERG responses of these patients, the amount of L and M cone activity has not been determined. ESCS patients have good acuity (in the absence of cystic maculopathy) and normal central color vision testing, which indicates that trichromatic cone function and circuitry must be present at least in the central macula. Evidence has been presented by Hood et al.3 that ESCS patients have an abnormally large number of S cones—estimated at upwards of 75 times the normal number (S cones are normally only a small percentage of the cone population). To make room for these cones, Hood et al. speculated that S cones developing earlier than rods may in some way limit the development of rods.

Using the new technique of multifocal electroretinography5 and focal electroretinography,6 we have investigated the topography of cone responses in one of the original patients from whom the ESCS was recognized and defined. The results give a 9-year follow-up on ERG abnormalities and show ERG differences between the central macula and the extramacular retina that have implications for the distribution of S, L, and M cones and their neural connections.

METHODS

This study conformed to the tenets of the Declaration of Helsinki. Both patient and control subjects gave informed consent, and the research was approved by our institutional Panel on Human Subjects in Medical Research. The subject of this study was the 19-year-old female whose unusual ERG findings led to the description of the ESCS.1,7 She was reexamined clinically and Goldmann fields obtained. Color vision was tested with the Farnsworth Panel D-15 test and the Lanthony Desaturated Panel D-15 test. Conventional ERG recordings were obtained with an LKC UTAS recording system (LKC Instruments, Gaithersburg, MD), using bipolar contact lens electrodes (Hansen Laboratories, Iowa City, IA, or Doran Instruments, Littleton, MA) and stimulus conditions that conformed to the International Society for Clinical Electrophysiology of Vision Standard for Electroretinography.8 The eyes were dilated with 1% tropicamide and 2.5% phenylephrine, and corneal anesthesia produced with 0.5% proparacaine. Focal ERGs were obtained with a Maculoscope (Doran Instruments), a modified direct ophthalmoscope that projects a bright annulus onto the retina, with a rapidly flickering core that elicits a focal cone ERG.6

The multifocal records were recorded with a VERIS recording system (Tomey Instruments, Nagoya, Japan, and ElectroDiagnostic Imaging, San Mateo, CA). Stimuli were presented on a high luminance monochrome monitor with a P104 phosphor, as a 105- or 241-element array of hexagonal cells. The pupils were dilated, and contact lenses were used as for the conventional ERG. The overall stimulated field measured ap-
proximately 50° in diameter. The multifocal stimulus was bi-
nary between a bright and a dark phase. In all experiments
stimulus contrast was higher than 98% measured on the
screen. Amplifier filter settings encompassed a band-pass of 1
to 300 Hz. The retinal response distributions to chromatic and
achromatic stimulations were compared. The bright phase of
the achromatic (white) stimulus was 200 candela per meters
squared (cd/m²). Red and blue stimuli were generated by
viewing the same display through Wratten 29 and 47B filters,
respectively. The bright phase of the red stimulus measured 15
cd/m² and that of the blue stimulus 5 cd/m². The identical
protocols were also run on a normal control subject, a 37-year-
old female with 20/20 visual acuity and no retinal disease.

In most recordings the pseudorandom multifocal stimulus
was updated at the video rate of 75 frames per second in
accordance with a binary m-sequence (here considered as a
sequence of + and −). When the element of binary m-se-
quence was +, the focal stimulus was bright and when it was
−, the focal stimulus was dark. In an attempt to distinguish the
on- and off-phase of the response the stimulation was slowed
down and modified as follows: The pseudorandom stimulus
was updated only after every 16 video frames. When the
element of binary m-sequence was +, the focal stimulation
was left in the bright state for 8 frames and in the dark state for the
second half of the base interval. When the controlling element
was −, the patch was dark for the entire base interval. Note,
however, that during its bright phase a stimulus patch was not
continuously bright. The short persistence screen phosphor
was scanned 8 times at 13.3-msec intervals. This resulted in 8
light flashes 13.3 msec apart. Although this flicker within the
central 50° of the visual field could not be resolved perceptu-
ally, it was reflected in the normal ERG response. Using a
television monitor for this mode of stimulation, one can only
roughly approximate the experimental condition of a contin-
uous long flash of light for the discrimination of the on- and
off-responses.

RESULTS

The conventional ERG of our ESCS patient showed no dark-
adapted rod response to a dim flash but a large, slow, rod-like
ERG waveform to a strong stimulus that was nearly identical
after dark- or light-adaptation (Fig. 1). This response has been
shown to have the spectral sensitivity of S cones. This full-field
ERG response was essentially unchanged over nearly 9 years
since her initial evaluation. There also have been no changes
in visual acuity (OD 20/25, OS 20/20), color vision (normal to
Panel D-15 tests), Goldmann visual fields (no scotomas or
constriction), or fundus appearance (stable sparse yellow le-
sions in the arcade regions).

Maculoscope recordings showed borderline normal ampli-
tudes (Fig. 2) when the 4° stimulus was centered on the fovea.
There was no response when the stimulus was centered 5°
off-center in the parafovea, but responses were obtained from
stimuli centered at the foveal edge (2° off-center).

Figure 3 shows a sample multifocal ERG stimulus array and
response arrays from both the ESCS patient and a normal
subject. The ESCS responses consisted mostly of monophasic “negative” waveforms except near the center of the macula. The dependence of ESCS responses on eccentricity can be seen better when responses from concentric rings of stimulus cells are averaged together. Figure 4 shows such ring averages for our ESCS patient and a normal subject. The central ESCS response rings (1–4) lack a normal b-wave peak near 28 msec, but have a peak near 35 msec. In the peripheral rings (6–9), however, the ESCS waveform is extremely prolonged, with a slow a-wave and with a b-wave peak near 60 msec.

We use the terms “a-wave” and “b-wave” here by convention to describe the initial negative, and subsequent positive, major waves of the multifocal ERG. The cellular origin of these waves may not be identical in multifocal and conventional ERGs, but there is evidence for a good deal of homology.9

Figure 5 shows ring-averaged multifocal ERGs to red and blue stimuli. The ESCS responses were in general of normal amplitude to red stimuli but larger by roughly a factor of two to blue stimuli. The central ESCS responses (rings 1 and 2) to red stimuli showed relatively normal b-wave peak timing (○), although the ESCS waveforms do not show the normal descent after the b-wave. The central ESCS responses to blue also had a relatively normal waveform but a somewhat more delayed b-wave peak at approximately 45 msec (●). A small residual peak, with the same time-to-peak as the response to red, is visible on the rising waveform (○). The peripheral ESCS responses (rings 4–6) were much slower than normal to both red and blue stimuli, with a prolonged a-wave and a delayed b-wave. The ESCS b-wave time-to-peak was much slower to blue light (●; almost 80 msec) than to red light (○; near 55 msec). A small hump with the timing of the red b-wave (near 55 msec) is seen with a blue stimulation (○).

**Table 1.**

<table>
<thead>
<tr>
<th>TEST AREA</th>
<th>PATIENT FIXATION</th>
<th>AMPLITUDE (μV)</th>
<th>ESCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fovea</td>
<td>0.18 – 0.39</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Foveal edge</td>
<td>---</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Parafovea</td>
<td>0.09 – 0.20</td>
<td>Nondetectable</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6 shows multifocal off-responses, produced with slow m-sequence blue-light stimulation and a long recording interval (see the Methods section) in a normal subject and in our ESCS patient. A small off-response from the central pixels was observed in our ESCS patient, which had latency similar to that of the normal subject. This response was not evident from rings beyond 7° off-center, even though the normal response grows in size. The oscillations that appear during the on-phase in the periphery of the normal subject are responses to the individual frames of the CRT display (13.33-msec intervals). The ERG of the ESCS patient did not follow this high frequency component of the stimulus with blue light stimulation.

**DISCUSSION**

The full-field ERG responses of our ESCS patient are the typical slow waveforms of this syndrome (indeed, this subject was one of the initial cases that defined this syndrome), and the lack of progression in 9 years is consistent with other reports that suggest a stationary or extremely slow progressive disorder.10 The normal multifocal ERG to white light shows little change in ERG waveform across the posterior pole. Our results show that the multifocal ERG in ESCS is characterized by a marked difference between responses from the central macula (within 5–7° of the foveal center) and those from more peripheral regions (7–20° eccentricity). In the central area the ESCS responses had a relatively normal waveform that was only modestly delayed in a-wave and b-wave time-to-peak. In the more peripheral regions the waveforms showed a striking prolongation of the a-wave, and the b-wave time-to-peak was much slower than normal. The peripheral responses to white light.
Figure 3. Multifocal ERG arrays. Top: Sample 241-stimulus cell array. During testing, each hexagon flickers on and off according to the m-sequence. The location of 20° eccentricity is shown. Middle: Topographic array of ERG responses in our ESCS patient. Note the slow negative waveform except in the center of the macula. Bottom: ERG array from a normal subject for comparison.
light match closely the appearance and timing of the full-field ERG in ESCS.

The results to colored stimulation are more complicated. The full-field ERG in ESCS shows much greater blue sensitivity than normal. However, in the areas of posterior retina covered by the multifocal ERG, we found blue responses to be only about twice normal in amplitude, whereas red responses were of normal size. In the central 7° of eccentricity, the ESCS responses to red and blue were relatively normal in waveform (a bit delayed to blue stimuli). But in the more peripheral areas (9–20° from center), the ESCS waveforms to both red and blue were very prolonged and there was a marked difference in b-wave time-to-peak between stimulation with red (50 msec) or blue (80 msec) light.

How can these findings be explained? In a normal eye S cones are absent in the central 100 µg of the foveola, but
constitute 1% to 2% of the cones in the fovea (with a density of 1–2000/mm²), and approximately 8% of cones at 4° from the center. If S cones increase to 75 times normal density and replace rods in ESCS, as suggested by Hood et al., there would be plenty of room for S cones outside the macula where rods can reach a maximum density of approximately 140,000/mm², and S cones are normally only 600/mm². However, there would not be enough room for a 75-fold increase in S cones added to the normal population of L and M cones in the fovea (with a peak density of 140,000 mm²). Any large increase in S cones in the foveal region would, to some degree, have to be at the expense of L and M cones.

ESCS patients have trichromatic cone function centrally because they have good acuity and color vision. However, the full-field cone ERG signal to red light or 30-Hz flicker is weak. One possibility is that ESCS patients have modestly reduced numbers of central L and M cones (perhaps 50% of normal, as suggested by the borderline maculoscope amplitudes), whereas the loss of peripheral L and M cones is more severe (perhaps to 10%–20% of normal) to account for the low full-field L and M cone ERG. An alternative possibility is that the peripheral L and M cone pathways are abnormal so that the b-wave responses are altered (as discussed further below). S-responding cones in ESCS may have a density roughly 75 times normal over most of the peripheral retina, but are perhaps only 20 to 30 times normal in the central macula to allow space for the retained L and M cones. These distributions would account in many respects for our ERG amplitude results. The central ERG in ESCS is more blue- than red-sensitive, because with blue stimuli there would be by this estimation 20 to 30 times more S cones than normally available to produce a response. However, the response to red light should represent L and M cones only, because the S cone spectral sensitivity curve does not reach significantly into the band-pass of the red Wratten 29 filter. We thus conclude that the peripheral response to red light owes its highly abnormal waveform to an abnormal L and M cone pathway in the ESCS patient. On the other hand, the spectral sensitivity of the M cones does extend into the band-pass of the blue Wratten 47B filter. This is confirmed by the relatively normal-looking L and M cone responses to blue stimulation of the normal eye. In the ESCS patient, however, blue stimulation produces additional waveforms that are not seen with the red stimulus and that we presume represent responses from the large numbers of S cones.

Figure 5. Ring responses in ESCS to red (left) and blue (right) stimuli. The data are presented as in Figure 4, with ring 1 the most central, and ring 6 the most peripheral (near 20° from fixation). The number of rings is smaller than in Figure 4 because these responses were recorded from an array of 103 rather than 241 stimulus cells to improve signal-to-noise ratio. The open symbols (○ and □) show the timing of b-wave peaks to red stimuli in the center and periphery, respectively. The filled symbols (● and ◆) show similarly the timing of b-wave peaks to blue stimuli. Normal responses, recorded under the same conditions, are superimposed as in Figure 4.
cones. The central b-wave has acquired a second peak at around 42 msec (●), and there is a large and broad late peripheral component peaking near 80 msec (●), to which we found no homologous feature in the normal eye.

The receptor potentials of L, M, and S cones have been shown to be essentially identical. Thus, the differences in b-wave waveforms between normal S cones and normal L and M cones must reflect differences in the pathway of these signals through the retinal circuitry. Although L and M cones feed onto on- and off-bipolar cells, both rods and S cones are believed to use predominantly an on-bipolar pathway. This may underlie why the normal S cone ERG is slower and more rod-like than the conventional L and M-dominated cone ERG. Alternatively, S cones may excite different systems of horizontal cells, other integrative cells, or both, which modulate and slow the development of the inner retinal potential changes that create the b-wave. Greenstein et al. have shown that the S-sensitive cells in ESCS are cones rather than rods. The normal trichromatic vision in ESCS suggests that L, M, and S cones centrally communicate through relatively normal pathways. Indeed, the normal S cone ERG has a time-to-peak of approximately 40 msec, which is similar to the 45-msec b-wave peak to blue light in the central rings of our ESCS patient. Outside of the central macula, however, this 45-msec peak diminishes rapidly and is overshadowed by a larger and slower response that peaks closer to 80 msec (which is long even for a rod response). It is conceivable that in the central macula of ESCS, the S cones use predominantly normal S cone pathways through the retina, whereas in the periphery where the massive numbers of S cones occupy space normally occupied by rods, the S cones communicate abnormally through rod pathways and generate an unusually slow b-wave response.

These explanations may also apply to the changes in a-wave time-to-peak between the center and periphery. However, some

**FIGURE 6.** Ring averages of blue-light multifocal ERG off-responses in our ESCS patient and a normal subject. Off-responses (indicated by the *dashed lines*) are seen clearly only in rings 1 and 2 of the ESCS patient, whereas they are prominent in all rings of the normal eye and increase in size with eccentricity.
of this apparent shift in a-wave time-to-peak may reflect the fact that a slower b-wave process in the periphery would be slower to reverse the negative response of the a-wave.

One puzzling aspect of our data is the difference in the timing of ESCS responses to red and blue light. In the central area, red light induces a rapid b-wave that is delayed only slightly relative to normal. This probably represents primarily a response of the L and M cones that are present centrally in ESCS but may include a contribution from the normal S cone ERG. However, it is harder to explain why the prominent ESCS response to red light in the periphery peaks near 55 msec, and thus is too slow for a normal S cone response but is more rapid than the peripheral (possibly rod-pathway) S cone response in ESCS. This difference in timing between red and blue peripheral responses in ESCS is unlikely to be a result of differences in the effectiveness of our red and blue stimuli on the S cone system alone, because the latter should not respond at all to the red light. It seems more likely that the peripheral L and M cones are in some way using or interacting with the peripheral S cone pathways, rod on-pathways, or both. Neural connections between different bipolar pathways are known at the amacrine cell level, although it is not known how they affect ERG recordings. The timing differences between red and blue peripheral responses may also reflect some aspect of the interactive mechanism that leads to the differences in b-wave timing between normal S and normal L and M cones, or reflect other differences in inner retinal pathways that are unknown at present. The white light waveforms in ESCS ( and in Fig. 4) have times-to-peak that are faster than the blue-stimulus times-to-peak. These may in part represent summations of the red and blue waveforms but may also reflect the tendency for b-waves to show faster times-to-peak with brighter stimuli. The responses from the normal eye were also faster to white stimuli, which in these experiments were 100-fold brighter than red and blue.

Some further support for these general speculations on cone distribution comes from our data on the on-off responses. Our data from the normal subject are consistent with the finding of Kondo et al. that the amplitude of the off-response, relative to the b-wave, increases with eccentricity. Our ESCS patient showed off-responses in the central retina that were close in latency to those from normal cones, but those in the peripheral retina seemed to be absent. This can be explained if off-responses from the center are dominated by the L and M cone circuitry, which appears to be functional in this region of the ESCS eye. Off-responses have been observed in the full-field ERG of some ESCS eyes, even though S cone pathways seem primarily to use on-bipolar cells. Our results suggest that regional differences may be relevant to understanding these observations.

References

Severe Ocular Abnormalities in C57BL/6 but Not in 129/Sv p53-Deficient Mice
Sakae Ikeda, Norman L. Hawes, Bo Chang, Cindy S. Avery, Richard S. Smith, and Patsy M. Nishina

Purpose. To demonstrate the importance of genetic background interaction on the development of ocular phenotypes in p53-deficient mice.

Methods. Eyes of adult mice, homozygous and heterozygous for the p53 gene disruption in the 129/SvJ and C57BL/6j (B6) genetic backgrounds, and their F1 progeny were examined by indirect ophthalmoscopy and by light microscopy.

Results. Indirect ophthalmoscopy revealed unilateral or bilateral vitreal opacities, fibrous retrolental tissue, and retinal folds in adult B6 mice but not in 129/Sv mice homozygous for a p53 null mutation. In B6 p53−/− mice, blood vessels extended from the peripapillary inner retina through the posterior vitreous and into the retrolental membrane. Optic nerves were hypoplastic.

Conclusions. These findings indicate that alleles from the B6 background contribute to the aberrant ocular phenotypes observed in p53 deficiency. They also suggest that p53 or the pathway in which it functions may be important for normal eye development. (Invest Ophthalm Vis Sci. 1999;40:1874–1878)

The Trp53 (p53) gene is best known for its role as a tumor-suppressor gene. More than half of tumors studied exhibit loss of heterozygosity or alterations in p53. It is also involved in the cellular response to stress including hypoxia, ionizing radiation, and teratogens. Cellular p53 response is mediated through transcriptional activation or direct signaling to regulate cell cycle control, differentiation, apoptosis, and/or angiogenesis (reviewed in Refs. 1–4). Yet, despite its apparent late cell cycle control, differentiation, apoptosis, and/or angiogenesis, much remains to be learned about its role in the retina.

In our exploration of the role of p53 in the apoptotic process of retinal degeneration, we found that p53−/− mice congenic on the C57BL/6j (B6) background have severe ocular abnormalities, whereas most 129/Sv p53−/− mice have normal fundus characteristics. In this report, we present a comparison of clinical and histologic effects of the p53 null mutation on ocular phenotypes in two genetic backgrounds, B6 and 129/Sv.

Materials and Methods

Animals

The p53 null mutation was prepared in strain 129-derived D3 ES cells, which were then microinjected into C57BL/6 blastocysts. The germ-line transformants were crossed either to C57BL/6 or a 129/Sv strain (R. Jaenisch); F1 mice were then intercrossed to produce homozygous null mutants. The 129/Sv-Trp53tm1Tyj line was imported to The Jackson Laboratory, and the mutation has been maintained by reciprocal backcrossing to three different genetic backgrounds: C57BL/6J (B6), BALB/cJ, and C3H/HeOuJ. The p53 null mutation has been backcrossed to B6 for 10 generations, and these mice are currently being intercrossed. For this study, 129/Sv-Trp53tm1Tyj (129/Sv p53−/−), congenic C57BL/6-Trp53tm1Tyj (B6 p53−/−); F1 homozygous null mutants (B6.129/Sv p53−/−), heterozygous (B6.129/Sv p53+/−) and wild-type (B6.129/Sv p53+/+) controls from a B6 p53−/− X 129/Sv p53+/− cross; and offspring from the backcross, F1(B6.129/Sv p53−/−) X B6 p53+/− or F1(B6.129/Sv p53−/−) X 129/Sv p53+/−, were obtained from The Jackson Laboratory Production Facility or were bred in our research colony. DNA was isolated from tail tips of mice and the IMR013, 5′-CCGGCTGCAGCGAGATTCGCTCTTCGT-3′; IMR014, 5′-AGTGGAAGATCGAGGAGATC-3′; IMR336, 5′-ATAGGGTCG-CCGGTTCAT-3′; and IMR337, 5′-CCCAGATATCTGGAAGAAGGGGTTG-3′ oligonucleotide primers were used in polymerase chain reaction (PCR) amplification to detect mice homozygous for the p53 null mutation. PCR products were resolved on a 3% metaphor/1% agarose gel and visualized with ethidium bromide staining. All mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Clinical Examination

Eyes of mice were dilated with atropine before examination by indirect ophthalmoscopy with a 60- or 78-D aspheric lens. Fundus photographs were taken with a Kowa fundus camera (Kowa Company, Tokyo, Japan) using a Volk superfield lens (Volk Optical, Mentor, OH) held 2 in. from the eye. The highest flash intensity was used with 400 ASA film.

Tissue Preparation

Adult mice were anesthetized with tribromoethanol and perfused with phosphate-buffered saline (PBS) followed by 1% paraformaldehyde-2% glutaraldehyde-0.1 M cacodylate. Enucleated eyes were stored in this ice-cold fixative for 24 hours, embedded in hydroxyethylmethacrylate, and sectioned in a plane to include the ora serrata and optic nerve. Alternatively, enucleated eyes were placed in Bouin’s fixative for 24 hours, embedded in paraffin, and sectioned as above. Sections were stained with hematoxylin and eosin. For optic nerve preparations, adult mice were killed by CO2 asphyxiation and then after removal of the brain, heads were placed in phosphate-buffered glutaraldehyde-paraformaldehyde for 48 hours and
transferred to 1 M phosphate buffer. The optic nerves extending from the orbits to the optic chiasm were dissected and postfixed with 1% osmium tetroxide, processed under standard procedures, and embedded in a 1:1 Epon–Araldite mixture. Cross sections cut at 1 μm were stained with 1% para-phenyldiamine in a 1:1 propanol-methanol mixture.

RESULTS

Incidence of Ocular Abnormalities in 129/Sv p53−/− and B6 p53−/− Mouse Stocks, Assessed by Clinical Examination

Thirteen homozygous null and 10 heterozygous 129/Sv-Trp53tm1Tyj mice examined by indirect ophthalmoscopy were normal. Ten B6 p53+/− and three B6 p53+/+ control mice were also examined, and no abnormalities were observed. However, of 65 B6 p53−/− mutants examined, 57 exhibited pigmented and nonpigmented fibrous retrolental tissue, vitreal opacities and/or retinal folds in both eyes, 5 mice were unilaterally affected, and 2 appeared normal by fundus examination but not by histologic examination. Cataracts were present in 14 of the 130 eyes examined. Of the ocular abnormalities, vitreal opacities and fibrous retrolental tissue were the most commonly observed (Fig. 1Left and Right). Aberrant ocular phenotypes were observed as early as 14 days of age, the earliest time point examined.

Histologic Examination

Both 129/Sv and B6 homozygous p53 null mutants had abnormally dilated peripheral retinal blood vessels. B6 p53−/− mice also exhibited abnormal vessels extending from the peripapillary inner retina (most often from vessels on the surface of the optic nerve) through the posterior vitreous and into the retrolental membranes. Both nonpigmented and pigmented membranes were observed. The pigmented retrolental membranes were presumably the result of migration of mobilized retinal pigment epithelial cells. Vitreous traction extending from the retinal surface or optic nerve toward the retrolental membrane was also observed (Fig. 2A). In severe cases, erosion of the posterior lens capsule with the beginnings of extrusion of lens cortex was often noted (Fig. 2B). The vitreal opacities revealed by fundus examination appeared to result from the accumulation of fibrous and vascular debris in the vitreous.

B6 p53−/− mice also exhibit unilateral or bilateral hypoplastic optic nerves. Examination of longitudinal sections of the retrolaminar optic nerve revealed fewer nerve fibers, as evidenced by the narrower columns between pial septae (Fig. 2C). In addition, the pial septae in many areas were disorganized when compared with similar sections from 129/Sv p53−/− or F1(B6/129Sv p53−/−) mice. Cross-sectional analysis of the optic nerves from B6 p53−/− mice at 10 weeks of age revealed optic nerve atrophy with reduction in the number of myelinated nerve fibers, some of which demonstrated degeneration (Figs. 2D, 2E). The degree of degeneration varied between optic nerves from the same animal and among animals of the same age.

The eyes of 129/Sv p53−/− mice examined were either normal or exhibited a thin blood vessel extending from the optic nerve head toward the lens. Although most of these vessels extended into the vitreous, a few reached the lens surface. No other abnormalities similar to those in B6 p53−/− mice were observed.

Genetics of the Ocular Phenotypes in B6-Trp53tm1Tyj Mice

To characterize the mode of inheritance of the ocular phenotypes, a number of crosses between mice homozygous or heterozygous for the p53 null mutation from the 129/Sv and B6 background were carried out. Fourteen of 17 F1 progeny homozygous for the p53 null mutation and all heterozygous progeny from a B6 p53+/− × 129/Sv p53−/− cross, exhibited the near-normal fundus characteristics of the 129/Sv p53−/− parental strain. The remaining three p53−/− F1 progeny exhibited either unilateral vitreal opacities, fibrous retrolental tissue, or retinal folds.

F1 progeny with no ocular abnormalities were then either backcrossed to the resistant 129/Sv-Trp53tm1Tyj or susceptible B6-Trp53tm1Tyj parental strain. In the F1(B6.129/Sv p53−/−) X 129/Sv p53−/− backcross, all 39 p53−/− backcross mice examined by indirect ophthalmoscopy were normal. However, although none of the heterozygous offspring in the F1(B6.129/Sv p53−/−) X B6 p53+/− backcross were affected, 26 of 30 p53−/− backcross offspring exhibited vitreal opacities, fibrous retrolental tissue, and/or retinal folds.

DISCUSSION

Ocular Abnormalities

Ocular abnormalities in B6 p53−/− mice are reminiscent of the histopathologic changes reported in human eyes with persistent hyperplastic primary vitreous (PHPV). Unilateral malformations including vitreal vasculature, retrolental tissue, and retinal fold and detachment are usually observed in these patients. In some cases, unilateral optic nerve head hypoplasia is also observed (reviewed in Ref. 9). No obvious cause or hereditary influence has been reported for human PHPV. The B6 p53−/− mouse may provide an excellent genetic animal model for human PHPV to study the mechanisms underlying this disease.

The vitreal vascularization observed in adult p53-deficient mice may in part be remnants of the hyaloid system that...
vascularizes the embryonic and early postnatal vitreous. This hyaloid vasculature normally regresses by adolescence, presumably through apoptosis and removal by intraocular macrophages known as hyalocytes. Although p53 is not necessary for all forms of apoptosis, indications are that apoptosis in the retina may be p53 dependent. p53 is thought to function early in the apoptotic pathway by downregulating bcl2 expression and by upregulating bax expression.
motes cell survival, whereas bax promotes cell death. Conceivably then, hyaloid vessels persist in p53-deficient mice because they never receive the proper p53-mediated signals to regress. In fact, it was recently reported that a delay in apoptosis of the hyaloid vasculature occurs in BALB/c p53−/− mice.13

Blood vessels originating from the optic nerve and from the retinal surface (ascertained by examination of serial sections through the eye), the proliferation of fibrous tissue in the vitreous and the hypoplastic optic nerve observed in B6 p53-deficient mice cannot be explained solely by p53’s effects on apoptosis. The centrally oriented vascularization, frequently reported in abnormal vascularization in human and animal eyes, poses the possibility that angiogenic factors normally under the control of p53 may diffuse toward the posterior pole of the eye.10 p53 is known to regulate two factors important in the balance of angiogenesis. In vitro, p53 deficiency leads to a reduction in thrombospondin 1, an antiangiogenic factor, and an induction of the potent mitogen, vascular endothelial growth factor (reviewed in ref. 17). In vivo, this sequence of events could lead to conditions favorable to endothelial cell proliferation and differentiation.

The hypoplastic optic nerve observed in B6 p53−/− mice appears to be caused by degeneration of rather than abnormal development of the optic nerve. The focal nature of the degeneration suggests that interaction of p53 with factors contained within these particular cells is necessary for their viability. When a severe degeneration of the optic nerve was observed, fewer cells were found in the peripheral ganglion cell layer in comparison with a normal eye (data not shown). The focal loss of the ganglion cell layer was limited to one well-defined retinal area, whereas the opposite side of the section demonstrated a normal number of cells. This observation is consistent with the focal degeneration of the optic nerve, because the ganglion cell axons originating from one region of the retina remain localized in a corresponding part of the optic nerve.18

A terminal dUTP nick-end labeling assay was performed in these eyes to determine whether the ganglion cells die by apoptosis. In five eyes from B6 p53−/− mice with focal optic nerve degeneration, no positively labeled cells were observed (data not shown). This suggests that cells either die by an alternate mechanism, or that the number of apoptotic nuclei at any given time point are so few that they are undetectable by this method. It is important to note that if cell death occurs through an apoptotic mechanism, it does not occur through the p53 pathway in these p53 null mutants.

Genetic Interaction and the Ocular Phenotypes in B6-Trp53<sup>1287</sup> mice

The effect of genetic background on the expression of phenotypes in p53 null mutants is not unprecedented; tumor type5 and incidence of embryonic exencephaly7 have been shown to be influenced by the strain on which the mutation is placed. Because vitreal vascularization, proliferation of fibrous tissue, and hypoplasia of the optic nerve were observed in B6 p53−/− mice but not to the same extent in 129/Sv p53−/− mice, we hypothesized that 129/Sv mice must have alleles that can compensate for the loss of p53 function, whereas B6 mice do not. Furthermore, that severe ocular abnormalities were observed only when the backcross was carried out with the B6 p53 null parent suggests that these phenotypes are caused by gene interactions of recessive B6 susceptibility alleles and that dominant 129/Sv alleles are protective.

The alternative explanation that the ocular abnormalities in the B6-Trp53<sup>1287</sup> mice are the result of a new spontaneous mutation independent of the p53 null mutation is an unlikely one. First, some of the abnormalities, although less severe, are also observed in the congenic stock derived from crossing 129/Sv p53−/− into the BALB/c background (now at backcross five). Second, similar phenotypic ocular abnormalities have been observed in an independently derived BALB/c p53−/− stock.11,13 Finally, if the observed abnormalities were caused by a new single-gene mutation, then we would have expected only 15 of 30 p53−/− mice to be affected in the F1(B6.129/Sv p53−/−) X B6 p53+/− backcross, rather than the 26 observed. The distribution of affected-to-unaffected mice in the B6 backcross suggests the interaction of multiple genes.

The phenotypic differences observed in p53-deficient mice in different background strains allows us the opportunity to perform a modifier screen to identify susceptibility and resistance alleles that interact with p53 in vivo. Such studies are currently under way.

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References

The Rhodopsin Content of Human Eyes

Anne B. Fulton,1 Janice Dodge,2 Ronald M. Hansen,1 and Theodore P. Williams2

PURPOSE. To measure the total amount of rhodopsin in human eyes across the life span and to test the hypothesis that the rhodopsin content of infants’ and the elderly’s eyes is lower than at other ages.

METHODS. Rhodopsin was extracted from retinal and pigment epithelial fractions of 196 eyes of 102 donors, ages 27 weeks’ gestation through 94 years, using quantitative procedures. To recover photopigment bleached by unavoidable light exposure, the fractions from 78 eyes were incubated with 9-cis retinal. The total photopigment (retinal plus pigment epithelial fractions) per eye was examined for significant changes with age, using the higher value from pairs of eyes.

RESULTS. The median rhodopsin content of the higher eye of adults is 6.45 nmoles (range, 3.33–10.84 nmoles) with 8 nmoles or more recovered from 28% of all adult eyes. The rhodopsin content of infants’ eyes (<12 months postterm) is significantly lower than that of older individuals and increases with age. After infancy, no change with age is found. For both infants and adults, 9-cis retinal significantly increases the amount of photopigment recovered without reducing the variance in the amount of photopigment recovered. The rhodopsin content is estimated to be 50% of the median adult amount early in infancy, approximately 5 weeks postterm (95% confidence interval, 0–10 weeks postterm).

CONCLUSIONS. A developmental increase in rhodopsin content occurs during infancy. Thereafter rhodopsin content remains constant. The amount of rhodopsin recovered from human eyes is quite variable. Bleaching alone cannot explain the variability. (Invest Ophthalmol Vis Sci. 1999; 40:1878–1883)

From 1The Department of Ophthalmology, Children’s Hospital and Harvard Medical School, Boston, Massachusetts; and 2The Biomedical Research Facility, Department of Biology, The Florida State University, Tallahassee, Florida.

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Reprint requests: Anne B. Fulton, Department of Ophthalmology, Children’s Hospital and Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115.

Development of the human rod outer segments (ROS) begins at preterm ages and continues with further elongation of the ROS after term.1 As in other species, it has been suspected that developmental elongation of human ROS, which proceeds after the addition of rod cells has ceased, is accompanied by an increase in rhodopsin content and scotopic retinal sensitivity. Previous measurements2,3 have indicated that the amount of rhodopsin in infants’ eyes is lower than in adults’, implying a developmental increase in rhodopsin content. However, a sufficient number of measurements have not been available to define the developmental course. After infancy, scotopic sensitivity remains constant4–6 until after the age of 60 years when slight deficits in scotopic sensitivity are found.7,8 It has been reasoned that the deficits in scotopic sensitivity at either end of the age span may be due to receptor or postreceptor factors, or a combination of the two.4,5,7,9,10

Thus, it is of interest to define the course of age-related changes in the rhodopsin content of the human eye. Since our previous reports,2,3 we have more than tripled the sample size and added a 9-cis retinal regeneration procedure to evaluate the effect of possible bleaching on rhodopsin content. The rhodopsin content of the eyes, ranging in age from the preterm weeks to more than 90 years, has been examined for significant changes with age.

METHODS

Eyes (n = 196) from 102 donors, 27 weeks’ gestation through 94 years of age, were obtained through eye banks, or in the case of the preterm infants, at autopsy. Data from 30 of these donors have been reported before.5 All globes appeared normal, and no donor had a history of eye problems except uncomplicated cataract surgery in three elderly donors.

As previously described,2,5 each globe was placed in a petri dish, containing 5 ml 0.9% saline, and bisected in an anteroposterior plane. The entire retina was teased free, placed in 5 ml distilled water, and vigorously stirred with a stainless steel spatula; this was designated the retinal fraction. The pigment epithelium and choroid were teased from the scleral shell, placed in a separate tube along with the saline from the petri dish and stirred vigorously; this was designated the pigment epithelial (PE) fraction. Each of these fractions was processed separately. The samples were centrifuged at 12,000g for 10 minutes at 4°C and the supernatant discarded.

Extraction of the photopigment was done with 1% CTAB (cetyl trimethyl-ammonium bromide; Sigma, St. Louis, MO),2,5 or 1% Emulphogene (Sigma) in 50 mM Tris-acetate buffer, pH 6.9. The results obtained with CTAB and Emulphogene are considered together in this report because the mean rhodopsin recovered from 20 pairs of adults’ eyes (CTAB mean = 8.19, SD = 1.63 nmoles); Emulphogene (mean = 6.67, SD = 1.99
nmol (final concentration, 10 nmoles/ml) for 1 hour at 20°C and then centrifuged at 12,000 g for 15 minutes. The supernatant was discarded, and 5 ml of detergent solution was added to each pellet. The pellets were disrupted with a spatula and incubated in the dark for 1 hour at 20°C, and spun again at 12,000g for 10 minutes. The 78 eyes included 37 for which the fellow eye was not incubated with 9-cis retinal, and only native rhodopsin (with 11-cis retinal) was assayed.

After the final spin, an aliquot of the supernatant was removed and scanned 820 to 190 nm using an HP-8452A diode array spectrophotometer to obtain the absorbance spectrum. The amount of photopigment in each eye. Extinction coefficients of 42,000 M⁻¹ cm⁻¹ for rhodopsin and 43,000 M⁻¹ cm⁻¹ for isorhodopsin were used. To determine whether isorhodopsin was present in the 9-cis retinal-supplemented samples, the method of wavelength shift²⁻¹⁻¹ was used. This technique makes use of the fact that isorhodopsin absorbs at shorter wavelengths (\(\lambda_{\text{max}} = 486\) nm) than does rhodopsin, and mixtures of the two photopigments produce a composite spectrum with a \(\lambda_{\text{max}}\) between those of the native rhodopsin and the artificially produced isorhodopsin.

The effect of age on the amount of photopigment recovered per eye was analyzed. Because light exposure and incomplete recovery of rhodopsin bearing tissues were possible explanations for recovery of spuriously low amounts of photopigment, but because artifactually high values were unlikely to result from the procedures described above, the higher amount of photopigment obtained from a pair of eyes was used for analysis of the effect of age. If only one eye was available (n = 8 donors), that eye was used in the analysis. A logistic growth curve⁵⁻¹⁵⁻¹⁶ of the form

\[ y/y_{\text{max}} = \frac{\text{age}^n}{(\text{age}^n + C^n)} \]

where C is the age at which \(y = 0\) of the adult value \(y_{\text{max}}\), provides a good summary of the course of rhodopsin increase during development in other species and was to be considered as a descriptor of human rhodopsin development.

**RESULTS**

The amounts of photopigment recovered from the 196 eyes are summarized in Table 1. The data from eyes having only native rhodopsin (opsin + 11-cis retinal) studied, and those having fractions incubated with 9-cis retinal, are listed separately. For all 108 adult eyes, 28% had 8 nmoles or more of native rhodopsin recovered. For all groups (Table 1), the amounts of photopigment recovered from eyes treated with 9-cis retinal overlap broadly and are analyzed further below.

For native rhodopsin, \(\lambda_{\text{max}}\) did not vary with age. The median value was 496 nm, and 90% of the values are within 2 nm of this value. Similar values (496–498 nm) have been reported previously for extracted human rhodopsin.¹⁷–²⁰ As the spectra in Figure 1 illustrate, the difference spectrum obtained from a 9-cis retinal–supplemented sample may be shifted to shorter wavelengths, indicating the presence of a mixture of isorhodopsin and rhodopsin rather than rhodopsin alone. The median \(\lambda_{\text{max}}\) for the 9-cis retinal–supplemented samples was 492 nm (range, 486–500 nm).

The distribution of \(\lambda_{\text{max}}\) values obtained from 9-cis retinal–supplemented and nonsupplemented eyes of the 57 paired

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Native Rhodopsin (nmoles)</th>
<th>9-cis Retinal Supplemented (nmoles)</th>
<th>Native Rhodopsin (nmoles)</th>
<th>9-cis Retinal Supplemented (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants</td>
<td>2.00</td>
<td>6.47</td>
<td>1.80</td>
<td>6.40, 6.88</td>
</tr>
<tr>
<td>(27 wk gest. to 11 mo)</td>
<td>(0.14–3.79)</td>
<td>(1.70–10.26)</td>
<td>(0.08–6.27)</td>
<td>—</td>
</tr>
<tr>
<td>(24 donors, 44 eyes)</td>
<td>n = 13⁺</td>
<td>n = 11⁺</td>
<td>n = 18</td>
<td>n = 2</td>
</tr>
<tr>
<td>Children</td>
<td>6.82</td>
<td>6.88</td>
<td>4.70</td>
<td>7.43</td>
</tr>
<tr>
<td>(1–13 years)</td>
<td>(2.10–7.98)</td>
<td>(1.45–8.81)</td>
<td>(1.31–6.78)</td>
<td>—</td>
</tr>
<tr>
<td>(13 donors, 25 eyes)</td>
<td>n = 5</td>
<td>n = 8</td>
<td>n = 11</td>
<td>n = 1</td>
</tr>
<tr>
<td>Adolescents</td>
<td>5.27</td>
<td>6.50</td>
<td>5.13</td>
<td>—</td>
</tr>
<tr>
<td>(13–21 years)</td>
<td>(1.72–8.99)</td>
<td>(4.01–9.81)</td>
<td>(0.14–7.24)</td>
<td>—</td>
</tr>
<tr>
<td>(10 donors, 19 eyes)</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 9</td>
<td>None</td>
</tr>
<tr>
<td>Adults</td>
<td>6.45</td>
<td>7.19</td>
<td>4.63</td>
<td>6.91</td>
</tr>
<tr>
<td>(21–94 years)</td>
<td>(3.33–10.84)</td>
<td>(2.33–10.50)</td>
<td>(2.55–9.22)</td>
<td>(2.55–9.70)</td>
</tr>
<tr>
<td>(55 donors, 108 eyes)</td>
<td>n = 23</td>
<td>n = 32</td>
<td>n = 34</td>
<td>n = 19</td>
</tr>
</tbody>
</table>

Values are median and range; number of donors and eyes.
* The infants in the 9-cis retinal–supplemented column (median, 3 months postterm; range, 27 weeks gestation to 10 months postterm) were, on average, older than those in the Native Rhodopsin column (median, 2 weeks postterm; range, 27 weeks gestation to 11 months postterm). The medians are not significantly different (Mann–Whitney U = 47; P = 0.11).
samples is compared in Figure 2. For the supplemented samples, there was a significant shift of the distribution to shorter wavelengths ($t = -5.43; df = 36; P < 0.01$). Among individual pairs ($n = 37$), supplementation achieved a large increment in photopigment in some, whereas there was no increase in others (range of differences between 9-cis retinal-supple-
mented and nonsupplemented samples: +5.96 nmoles higher to -0.41 nmoles lower. The increment in amount of photopigment recovered was significantly correlated with that of nanometers that $\lambda_{\text{max}}$ was shifted, from 496 nm to shorter wavelengths ($r = 0.41; P < 0.02$). The increment for 9-cis retinal treatment is similar in infant and older donors. For the infants ($n = 9$), the amount recovered from the nonsupplemented eye (mean = 3.23; SD = 2.46 nmoles) was 66% of that recovered from the supplemented eye (mean = 4.87; SD = 2.57 nmoles). For the older donors (2–94 years; $n = 28$), the amount recovered from the nonsupplemented eye (mean = 4.42; SD = 2.26 nmoles) was 70% of that recovered from the supplemented eye (mean = 6.28; SD = 2.08 nmoles). Despite achieving the expected increment in photopigment by 9-cis retinal supplementation, the variability in the amount of photopigment recovered was not significantly reduced. There was no significant difference in the variance of the supplemented and nonsupplemented samples ($F = 1.18; df = 36.36; NS$).

The normalized, photopigment content of the 102 donors is shown as a function of age in Figure 3. For donors contributing pairs of eyes, the result from the eye with the higher amount of photopigment is shown. Only during infancy is there a significant change in the amount photopigment recovered ($y = 2.178 \times \text{age}^0.5$), where $y_{\text{max}} = 100\%$, $n = 7.2$, and $C = 5$ weeks, the age at which rhodopsin is 50% of the adult value.
photopigment in supplemented samples was 7.13 (3.55–10.1) nmols. According to the growth curve shown in Figure 3, the age at which rhodopsin content of the eye is 50% of that in adults is 5 weeks (95% confidence interval, 0–10 weeks post-term).

**Discussion**

These data show that the rhodopsin content of infants’ eyes is lower than in adults and that the rhodopsin content increases significantly during infancy, when a developmental increase in visual sensitivity occurs.9,10 Thus, a lower amount of rhodopsin, and consequent lower probability of photon capture by rhodopsin in the outer segments, cannot be disregarded as one of the fundamental determinants of infants’ lower scotopic visual sensitivity. At the other end of the life span, although loss of photoreceptors and quantum catching capability were considered as possibly contributing to an average deficit of 0.5 log unit in scotopic visual sensitivity9 and 0.2 log unit deficit in scotopic b-wave sensitivity4 in older observers, postreceptorial factors appeared more likely. The rhodopsin data of the present study are consistent with this conclusion.

The amounts of pigment recovered are quite variable at all ages (Table 1; Fig. 3). Some of the variability is likely due to bleaching of rhodopsin around the time that the eyes were procured. This supposition is consistent with the higher amount of photopigment found in eyes treated with 9-cis retinal. However, even among the 9-cis retinal-supplemented samples from adults, the standard deviation for nanomoles of photopigment recovered is approximately 33% of the mean, a little lower than that for the nonsupplemented samples for which the standard deviation is nearly 50% of the mean. However, even 33% is higher than the standard deviation typically obtained in laboratory experiments using the same type of extraction and regeneration procedures as used herein.14,21 Possibly, the regeneration achieved with the 9-cis retinal procedure in human retinas is less complete than in laboratory experiments, although control experiments did not indicate this to be the case. Thus, in human eyes, the variation in rhodopsin content may be controlled not only by the acute light history but also by other factors. For example, from retina to retina there is some variation in the number of rods present. Curcio and coworkers22 report that the number of rods in some retinas may be more than human retina ranges from 77.9 to 107.3 million. In other assays of rhodopsin, the difference between the rhodopsin content of infants and young adult eyes is 5 weeks (95% confidence interval, 0–10 weeks post-term).

Despite the variability that appears in these quantitative assays of rhodopsin, the difference between the rhodopsin content in infants and adults is significant. Surely this must accompany the development of ROS structure and function.

**References**


The Course of Maturation of Rod-Mediated Visual Thresholds in Infants

Ronald M. Hansen and Anne B. Fulton

PURPOSE. To measure the developmental course of infants' rod-mediated thresholds.

METHODS. Thresholds for detecting stimuli (2° diameter, 50 msec duration) presented at 10° (parafoveal site) or 30° (peripheral site) from a central fixation target were estimated using a preferential-looking method. Nine infants were tested at both stimulus positions at ages 10, 18, and 26 weeks.

RESULTS. At 10 weeks, infants' thresholds at both sites were significantly higher than those of adults. The infants' average threshold at 10° was 0.5 log unit higher than the infants' average threshold at 30°. Adults' thresholds at the two sites were equal. Thresholds of all infants decreased with age until by age 26 weeks the parafoveal and peripheral thresholds were equal and were the same as those of adults. The rate of change of parafoveal thresholds was significantly faster than the rate of change of peripheral thresholds.

CONCLUSIONS. Although postreceptoral factors cannot be ruled out, the results suggest that developmental increases in rod outer segment length and rhodopsin density account for most of the threshold changes during infancy.


The dark adapted, rod-mediated visual thresholds of normal infants are significantly higher than those of adults.1–9 For example, cross-sectional data show that the average threshold is 1.4 log unit higher than that of adults at 4 weeks, 1.1 log unit higher at 10 weeks, and 0.65 log unit higher at 18 weeks.7 Thus, although thresholds decrease at age 18 weeks they remain significantly above those of adults.9 The age at which infants' thresholds become equal to those of adults is unknown. Furthermore, we are unaware of any report that describes the course of maturation of normal scotopic thresholds in individual infants.

We undertook a longitudinal study of rod-mediated threshold development and elected to test parafoveal (10° eccentric) and peripheral (30° eccentric) retinal sites, because development is nonuniform across the retina. For instance, anatomic studies show parafoveal rod photoreceptor outer segment growth is delayed relative to peripheral outer segment growth,10–13 despite the axiom that the central retina matures earlier than the peripheral retina. Psychophysical study shows that 10-week-old infants' thresholds at a parafoveal site were significantly elevated relative to their thresholds at a peripheral site, whereas adults' thresholds at these sites were equal.14 In this longitudinal study, we tested the hypothesis that thresholds measured at the parafoveal site change more rapidly than those at the peripheral site.

METHODS

Stimuli

Stimuli were 50 msec, 2° diameter, blue spots (Wratten 47B, λ < 510 nm) presented on a rear projection screen, 10° or 30° to the right or the left, of a 30-min arc diameter red LED fixation target flickering at 1 Hz. Stimulus intensity was controlled by calibrated neutral density filters. Calculation of the retinal illuminance produced by the stimuli was based on luminance measurements made with a calibrated photodiode (UDT S-350; United Detector Technology, Orlando, FL) placed in the position of the subject's eyes. At the beginning and end of each session, the subject's pupillary diameter was estimated by direct observation with an infrared viewer. Pupillary diameter was determined by comparison with the diameter of the cornea which is 11 ± 0.5 mm in infants from term to 6 months of age.15 Retinal illuminance varies directly with pupillary diameter and the transmissivity of the ocular media and inversely with the square of the posterior nodal distance.7 The scotopic troland value of the stimulus16 was calculated taking each subject's measured pupillary diameter and the average axial length17 into account.6,7 The correction for light losses in the ocular media was based on previous results in infants.18

Procedure

Thresholds were estimated using a two-alternative, forced-choice, preferential-looking method19 that incorporated a fix-and-flash procedure.9 After the subject dark adapted for 30 minutes, an adult held the infant 50 cm in front of the center of the screen. The flickering red LED fixation target attracted the infant's gaze to the center of the screen. A second adult watched the infant with an infrared viewer and reported when the infant was alert and looking at the fixation target. The
fixation target was extinguished, and a test flash presented. The observer reported stimulus location, right or left, based on the infant’s head and eye movements and received feedback on every trial. Threshold was measured with a transformed up-down staircase that estimated the 70.7% correct point of the psychometric function. Control experiments with adults indicated that the observer could reliably detect a horizontal deviation of 3° or more from the fixation target. Thus, a reliable response to the 10° eccentric stimulus was expected.

Each infant was tested first at age 10 weeks, the age at which previous research has shown that thresholds are significantly higher for the stimuli at 10° than those at 30°, and thresholds at both eccentricities are significantly higher than those of adults. The second visit was scheduled at age 18 weeks because an earlier study showed that thresholds for 50 msec, 10° diameter stimuli presented 20° from fixation remained significantly higher than those of adults. As the 18-week-old infants’ results were collected, it was clear that thresholds for the 2° stimuli remained above those of adults at both sites.

Additional sessions at 8-week intervals were planned to continue until thresholds at both sites reached the adult troland value. Consequently, the infants were next scheduled to return at age 26 weeks. At every session, thresholds were obtained from each infant at both eccentricities (10° and 30°). At each age, five of the subjects were tested first at 10°, and four were tested first at 30°.

Normal adult control subjects were tested using the same 2° diameter stimuli at 10° and 30° eccentricities in conjunction with the staircase procedure. Adults named the position (left or right) of the stimuli.

Subjects
Fifteen infants aged 69 to 81 days (median, 71 days) at the first session participated. Nine completed testing at all three ages (10, 18, and 26 weeks); their data are the basis for this report. Six did not complete testing, and their data are not included. Two infants did not complete the first session, three completed only the first session, and one completed the first and second sessions, but not the third. The thresholds obtained from these infants are within the range of those in infants who completed the longitudinal study.

All infants were born within 10 days of term, were in good general health, and had normal eyes documented on thorough ophthalmic examination. Fifteen adults (aged 19–35 years) with normal eyes were tested; their results have been reported previously. The study conformed to the tenets of the Declaration of Helsinki and was approved by the Children’s Hospital Committee on Clinical Investigation. Written, informed consent was obtained from the infants’ parents and from adult subjects before each session.

Results
The thresholds of individual infants are shown in Figure 1. At age 10 weeks, all thresholds at both eccentricities were higher than any of the adults’ thresholds. The difference between the median thresholds of infants and adults at 10° was 1.06 log unit (Mann–Whitney = 0; P < 0.01) and at 30° was 0.58 log unit (Mann–Whitney = 0; P < 0.01). Previous studies of infants’ and adults’ spectral sensitivities indicate that thresholds with these troland values are rod mediated. These results agree well with the parafoveal and peripheral thresholds in eleven 10-week-old infants previously reported.

Thresholds decreased with age at both 10° and 30° sites in every subject (Fig. 1, upper and middle panels). At age 18 weeks, thresholds remained immature; the median thresholds at 10° and 30° were 0.62 and 0.29 log unit higher than the median threshold of adults. By age 26 weeks, the threshold of every infant at both 10° and 30° was within the adult range. Analysis of variance with repeated measures on two factors (age and target location) showed significant effects for age (F = 296; df 2,8; P < 0.01) and target location (F = 131; df 1,8; P < 0.01) and a significant interaction of age and target location (F = 53; df 2,8; P < 0.01).

The rate of change in threshold was more rapid at 10° than at 30° (Fig. 1). Between ages 10 and 26 weeks, thresholds decreased, an average of 1 log unit at the 10° eccentricity. Assuming a linear decrease in log threshold, at 10° the median rate of change, determined by linear regression of each infant’s data, was 0.067 log unit per week (range, 0.053–0.080 log unit). At 30° the median rate was 0.035 log unit per week (range, 0.023–0.044 log unit). The rate of change at 10° was
significantly faster than at 30° (Wilcoxon matched-pairs signed-rank test; $T = 0; P < 0.01$).

The difference between thresholds at 10° and 50° ($\Delta_{10-50}$) decreased after age 10 weeks (Fig. 1, bottom). By age 26 weeks all infants had $\Delta_{10-50}$ values within the range found in adults.

**DISCUSSION**

The rates of change in parafoveal and peripheral thresholds were not equal. Parafoveal thresholds, which at age 10 weeks are less mature than peripheral thresholds, changed at twice the rate of peripheral thresholds (Fig. 1). Dark-adapted thresholds for small, brief stimuli obtained in this within-subject study, were unequal at parafoveal and peripheral sites in 10-week-old infants. This difference decreased systematically in every infant and vanished by age 26 weeks. Moreover, by 26 weeks, thresholds at both sites equaled those of adults.

The developmental course for the parafoveal (10° eccentric) thresholds (Fig. 1, top) was similar to that for the cross-sectional data. The cross-sectional studies were performed with stimuli more than 2° in diameter presented at approximately 20° eccentricity. At age 8 to 10 weeks, the dark adapted thresholds are 1.1 to 1.5 log unit higher than those of adults. All these previous studies were performed with larger diameter stimuli than the 2° used in the present study. In the present study the median parafoveal threshold at 10 weeks was 1.06 log unit higher than the median adult threshold. At 18 weeks, Hansen et al. found that dark-adapted thresholds are elevated by 0.65 log unit, which agrees well with the median threshold elevation (0.62 log unit) found in the present study.

The troland value of the 2°, 50 msec stimulus takes into account pupillary diameter, posterior nodal distance, and light losses in the media of the eye. With such large stimuli, in dark-adapted conditions, scotopic visual efficiency is high and is limited by photoreceptors and postreceptorial pooling rather than by optical or preretinal factors.

Changes in rod outer segment (ROS) length and rhodopsin density can account for the improvement in rod-mediated thresholds at parafoveal and peripheral sites. Anatomic development of the retina continues during the ages over which thresholds have been measured. ROSs increase in length. Thus, the increased probability of photon capture must be considered among the possible explanations for the observed decreases in thresholds. Are thresholds proportional to ROS length?

Measurement of peripheral rod outer segments illustrated in Figures 41-13 and 41-14 of Hendrickson indicate that peripheral ROS length in adults are 2.3 times longer than those of 5-day-old infants, predicting a threshold difference of approximately 0.4 log unit. In the present study, the median 10-week-old infant threshold at the peripheral site was 0.58 log unit (range, 0.4-0.85) higher than the median adult threshold. Also of note, the peripheral retinal sensitivity of 10-week-old infants, estimated with the full-field scotopic electroretinogram, is also approximately 0.5 log unit less than that of adults. Elevations of 0.5 and 0.58 log unit are a bit higher than expected from ROS lengths alone. However, in developing ROSs, at least in infant rats, ROS length overestimates the amount of rhodopsin available for photon capture.

At the parafoveal site, ROS length in an adult is nine times that of a 5-day-old infant, which predicts a threshold difference of nearly a log unit. At age 10 weeks, the difference between the median infant and adult thresholds at the parafoveal site is 1.06 log unit (range, 0.85-1.3). At age 11 months, the next age studied anatomically, the parafoveal and peripheral ROS lengths of infants are 69% and 67% of adult ROSs. These ROS lengths predict thresholds differences of only 0.16 and 0.17 log unit, which may not be detectable across subjects. The present psychophysical data show that no infant has a threshold that is more than 0.1 log unit different from the median adult threshold by age 6 months. Thus, at both peripheral and parafoveal sites, the increased probability of photon capture associated with increased ROS length helps explain threshold changes during infancy.

ROS lengths in the parafovea and periphery were also compared. At age 5 days after term, parafoveal ROS lengths are only approximately a quarter of those in more peripheral retina. This predicts that the parafoveal threshold is four times, or 0.6 log unit, higher than the peripheral threshold. In 10-week-old infants, the median parafoveal threshold was 0.5 log unit higher than at the peripheral site (Fig. 1). At age 11 months after term, both parafoveal and peripheral ROS have elongated and are equal in length, predicting equal thresholds at these sites by age 11 months. In fact, based on the thresholds (Fig. 1), we suspect parafoveal and peripheral ROS lengths are equal by age 6 months.

In summary, during development, the rate of change of rod thresholds is significantly higher in parafoveal than peripheral retina. The differences between infant and adult scotopic thresholds are reasonably well accounted for by photoreceptor outer segment immaturities. Although immature postreceptorial processes such as the functional organization of receptive fields, which are thought to underlie spatial summation and the gain of transmission from photoreceptors to second-order neurons are acknowledged, the hypothesis that photoreceptor immaturities explain the scotopic thresholds of infants cannot be rejected. Studies of background adaptation can test this hypothesis by comparing shifts of the eigengrau and dark-adapted thresholds of increment thresholds measured in parafoveal and peripheral retina.

**Acknowledgments**

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**References**


